INDIAN AGRICULTURAL RESEARCH INSTITUTE (PUSA)

IARI

जैव-जीवनकृषि

LIBRARY

New Delhi

Call No

Acc No 25081

STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME 84

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1933

Made in the United States of America

Results of the investigations conducted at The Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is Volume 84. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, volume, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.

COPYRIGHT, 1933, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS
BALTIMORE, U. S. A.

CONTENTS

THE DEPARTMENT OF THE LABORATORIES

Pathology and Bacteriology

PAGE

SMITH, FREDERICK, and DICK, MACDONALD. The influence of the plasma colloids on the gradient of capillary permeability... 1

LANDSTEINER, K., and VAN DER SCHEER, J. Serological reactions with simple chemical compounds (precipitin reactions)..... 21

MILLER, FRANKLIN R. The induced development of non-acid-fast forms of *Bacillus tuberculosis* and other mycobacteria.. 33

FAVILLI, GIOVANNI. The influence of some organ extracts on the permeability to water of sea urchin eggs (*Arbacia punctulata*). 47

CASEY, ALBERT E., and ROSAHN, PAUL D. Supravital differential counting adapted to clinical use...................... 57

MURPHY, JAMES B., and STURM, ERNEST. Properties of the causative agent of a chicken tumor. VI. Action of the associated inhibitor on mouse tumors............................ 61

MURPHY, JAMES B., and STURM, ERNEST. Properties of the causative agent of a chicken tumor. VII. Separation of the associated inhibitor from tumor extracts..................... 71

STURM, ERNEST, and DURAN-REYNALS, F. Properties of the causative agent of a chicken tumor. VIII. The effect of testicle extract on the rate of growth of Chicken Tumor I......... 77

FLEXNER, SIMON. Immunity to human and passage poliomyelitis virus.. 85

Chemistry

LEVENE, P. A., STEIGER, ROBERT E., and ROTHEN, ALEXANDRE. The relation of chemical structure to the rate of hydrolysis of peptides. VII. Hydrolysis of dipeptides by alkali......... 93

LEVENE, P. A., and STEIGER, ROBERT E. Studies on racemization. XI. Action of alkali on polypeptides............... 103

PAGE
LEVENE, P. A., and RAYMOND, ALBERT L. On the structure of the so called 5-methylglucose of Ohle and von Vargha..... 115
LEVENE, P. A., and RAYMOND, ALBERT L. The substitution of glucose in position (4). I........................ 127
LEVENE, P. A., and MARKER, R. E. Maximum rotations and correlation of disubstituted acetic acids containing a methyl group.. 141
LEVENE, P. A., and HARRIS, STANTON A. The ribosephosphoric acid from xanthylic acid. II............................ 149
LEVENE, P. A., and CORTESE, FRANK. A note on the use of 1-bromotetramethylglucose for the synthesis of methylated glycosides.. 157
LIPMANN, FRITZ A., and LEVENE, P. A. Serinephosphoric acid obtained on hydrolysis of vitellinic acid.................. 161
LEVENE, P. A., and WALTI, A. Phytochemical reduction of 1-hydroxy-2-oxo-heptane (heptanol-1-one-2)............... 167

Chemical Pharmacology

JACOBS, WALTER A., and ELDERFIELD, ROBERT C. Strophanthin. XXVII. Ring III of strophanthidin and related aglucones.. 171
JACOBS, WALTER A. The ergot alkaloids. I. The oxidation of ergotinine.. 183

Experimental Surgery

PARKER, RAYMOND C. The functional characteristics of nine races of fibroblasts................................... 189
PARKER, RAYMOND C. The stability of functionally distinct races of fibroblasts................................... 193

General Physiology

NORTHROP, JOHN H. Crystalline pepsin..................... 197
NORTHROP, JOHN H. Crystalline pepsin. IV. Hydrolysis and inactivation by acid................................... 227
NORTHROP, JOHN H. Pepsin activity units and methods for determining peptic activity............................. 235
ANSON, M. L., and MIRSKY, A. E. The estimation of pepsin with hemoglobin.. 253

PAGE

BLINKS, L. R. Protoplasmic potentials in *Halicystis*. II. The effects of potassium on two species with different saps...... 259

OSTERHOUT, W. J. V. The kinetics of penetration. IV. Diffusion against a growing potential gradient in models........ 269

Physical Chemistry

MICHAELIS, L. Ein Reduktions-Indikator im Potentialbereich der Wasserstoffüberspannung.............................. 277

SCHUBERT, MAXWELL. Complex types involved in the catalytic oxidation of thiol acids.................................. 281

THE DEPARTMENT OF THE HOSPITAL

GOODNER, KENNETH, and DUBOS, RENÉ. Studies on the quantitative action of a specific enzyme in Type III pneumococcus dermal infection in rabbits.............................. 293

SPRUNT, DOUGLAS H. Infectious myxomatosis (Sanarelli) in pregnant rabbits... 303

PAGE, IRVINE H., and ALVING, ALF. The effect of nasopharyngeal operations on renal function......................... 311

VAN SLYKE, DONALD D., ALVING, ALF, and ROSE, W. B. Studies of urea excretion. VII. The effects of posture and exercise on urea excretion... 329

PAGE, IRVINE H., and VAN SLYKE, DONALD D. A simple test for plasma protein contents below the edema-producing level... 343

COHN, ALFRED E., and STEELE, J. MURRAY. Studies on the effect of the action of digitalis on the output of blood from the heart. I. The effect on the output of the dog's heart in heart-lung preparations.. 345

STEWART, HAROLD J., and COHN, ALFRED E. Studies on the effect of the action of digitalis on the output of blood from the heart. II. The effect on the output of the hearts of dogs subject to artificial auricular fibrillation.................. 375

STEWART, HAROLD J., and COHN, ALFRED E. Studies on the effect of the action of digitalis on the output of blood from the heart. III. Part 1. The effect on the output in normal human hearts. Part 2. The effect on the output of hearts in heart failure with congestion, in human beings.......... 397

PAGE

ANTHONY, ALBERT J., COHN, ALFRED E., and STEELE, J. MURRAY. Studies of the influence of pulmonary motion and distention on movements of the thorax. I. Chemical and nervous factors involved in establishing apnea. II. The relation between pulmonary and thoracic movements.............. 445

ANTHONY, ALBERT J., COHN, ALFRED E., and STEELE, J. MURRAY. Studies on Cheyne-Stokes respiration...................... 465

THE DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY

SEIFRIED, O., and CAIN, C. B. Histological studies on hog cholera. II. Lesions of the vascular system.................. 489

SEIFRIED, O., and CAIN, C. B. Histological studies on hog cholera. III. Lesions in the various organs............. 495

SHOPE, RICHARD E. Studies on immunity to swine influenza..... 507

STOLL, NORMAN R. Note on reinfection under "natural" conditions with a gut nematode of the rabbit................. 519

STOLL, NORMAN R. Studies with the strongyloid nematode, *Haemonchus contortus*. II. Potential infestation curves under conditions of natural reinfection......................... 527

SARLES, MERRITT P. Development of an acquired resistance in rabbits by repeated infection with an intestinal nematode, *Trichostrongylus calcaratus* Ransom, 1911................ 545

INDEX TO VOLUME 84....................................... 571

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1932,
Vol. 56, No. 3, pp. 371–389]

THE INFLUENCE OF THE PLASMA COLLOIDS ON THE GRADIENT OF CAPILLARY PERMEABILITY

BY FREDERICK SMITH, M.B., B.CH., AND MACDONALD DICK, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, April 27, 1932)

The wall of the capillary has long been considered on good evidence as behaving like an indifferent semipermeable membrane separating the blood from the tissues. All along the course of the little vessel the permeability has been supposedly the same, differences in the happenings at various points being referable to the interplay of several factors, diffusion, hydrostatic pressure, and osmosis chief among them. It has been held that in the first portion of the capillary the force of hydrostatic pressure dominates over the osmotic pressure of the blood colloids, with the result that fluid is forced out into the tissue to be reabsorbed again as the vein is approached, because the drawing power of the colloids there prevails. The recognition that the permeability of the capillaries of some organs—muscle, skin—increases progressively in the direction of flow (1) has necessitated a reconsideration of the rôle of the factors just referred to. Recent experiments have shown that the gradient of capillary permeability exists independently of the hydrostatic pressure (2). The present work was undertaken to disclose the relation to it of osmotic conditions and especially of the influence of the osmotic pressure of the blood colloids.

Methods

The effect on the gradient of capillary permeability of greatly modifying the percentage of the circulating blood proteins has been directly followed. For this purpose several methods were employed.

Adult rabbits of about 2000 gm., fasted 24 hours but with access to water, were used throughout. They were anesthetized with sodium iso-amyl-ethyl barbiturate (sodium amytal—Eli Lilly and Co.) given intravenously, 0.5 to 0.7 cc. of 10 per cent solution per kilo. Freshly prepared solutions were always employed. For surgical procedures light ether was required in addition. Loss of heat was prevented by keeping the animal on a warm pad.

Chicago blue 6B was the dye of choice, and voluntary muscle the tissue observed. As proven in a previous paper (1) specific affinities are not responsible for the staining, for the dye passes directly from the blood to the interstitial fluid, the initial coloration of the muscle being due solely to its presence in this fluid. It is poorly diffusible and the gradient of its escape is plainly visible in the gross color pattern. The initial escape of the dye, which is restricted to the further portion of the capillaries, can be followed with the microscope.

The warmed isotonic solution of purified Chicago blue (7 per cent in 0.45 per cent NaCl) was introduced in approximately 3 minutes; 7 minutes later the carotids were cut and the muscles examined at once. The general technique of examination has already been described, as also the color pattern indicative of the gradient of permeability along the muscle capillaries (1),—regularly distributed blue bars in long muscles, and a fern-like pattern in the sheet muscles of the abdomen, attesting to the progressive increase in the escape of dye along the further part of the capillaries. Variations in the rate and abundance of escape are frequent in normal animals; and hence only marked and constant departures from the characteristic findings can be deemed significant.

Some of the experimental procedures entailed important changes in the blood volume. The cell-plasma ratio furnished a rough index to them. To obtain this ratio duplicate blood specimens taken from the ear into standard 0.04 cc. pipettes were diluted with 2 cc. of 0.9 per cent NaCl in Hamburger tubes (3) and the proportion of cells determined after centrifugation. By this method changes in the cell volume due to alterations in the tonicity of the serum were avoided. In all calculations the normal blood volume of the rabbit was assumed to be 5.5 per cent of the body weight (4).

The carotid blood pressure was followed with a kymograph. Blood proteins were determined by the method of Van Slyke, using specimens taken from an ear vein. In certain cases the percentages of both albumin and globulin were determined in order to apply the osmotic pressure formula of Govaerts (5).

Effects of Increasing the Plasma Proteins

The recent work of Adolph and Lepore (6) has shown that during the readjustment of blood bulk after introduction of hypo- and hypertonic solutions into the circulation, the voluntary muscles gain and lose fluid respectively. It is well known that the plasma constituents of transfused blood begin to leave the circulation almost at once, and it seems not improbable that some portion enters the muscle. If this is the case, the conditions affecting distribution of dye to this tissue during the readjustment after transfusion may be very different from those obtaining when the blood bulk has become stationary. For this reason we have in some instances injected dye after the readjustment had been accomplished and in others while it was still in progress.

By the intravenous injection of large amounts of compatible heparinized plasma, or in some instances of serum, it proved possible to increase the concentration of the plasma proteins by as much as 40 per cent. This came about through a greater intravascular retention of protein than of the other plasma constituents. The procedure involved a considerable though transitory increase in blood volume. With the aim of avoiding this we worked out a method whereby the proteins of the blood could be concentrated *in vitro* for injection.

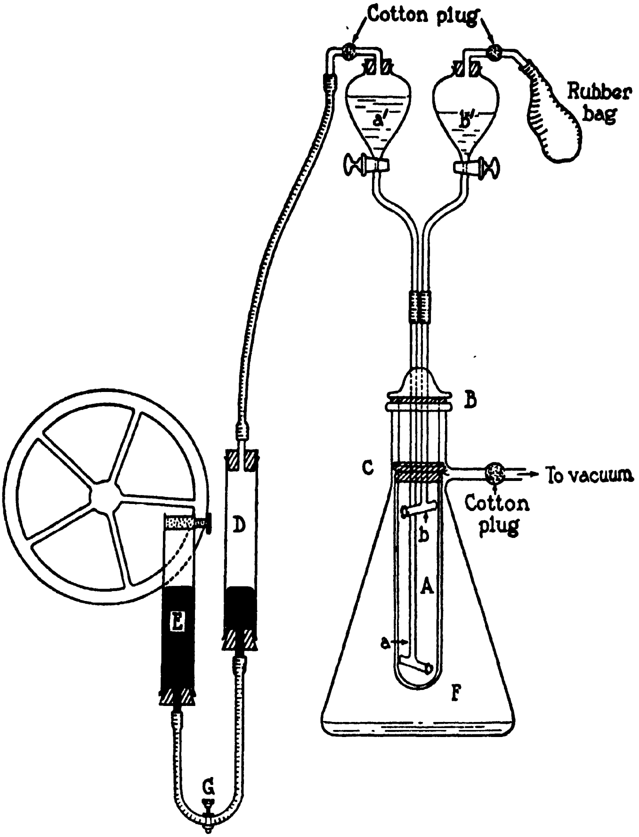
Concentration of the Blood Proteins

The fresh sterile sera of a number of rabbits are pooled and introduced into a specially devised concentrator.

A is a broad glass tube about 4 cm. in diameter and 18 cm. long, with one end rounded and the other expanded into a cap which rests at its edge on a ring of rubber (*B*), thus closing the filter flask (*F*) into which the tube is introduced. Two small tubes, *a* and *b*, pass down through the large one to end in cross-tubes which, extending through its wall, open near the top and bottom respectively. They terminate above in the bulbs *a′* and *b′*, the serum reservoirs. All the connections with *A* are sealed in.

A collodion tube slightly larger than the glass one, made according to Brown's (7) method, and just retaining proteins while letting crystalloids through freely ("80 per cent membrane"), is treated with 80 per cent alcohol and tied over *A* upon a rubber ring *C*. A close-fitting gauze jacket affords it support, preventing rupture when the pressure is lowered in the filter flask. This jacket does not appear in the diagram. The gauze has been previously sterilized and shrunken by boiling. About 2 mm. clearance should exist between the glass tube and the collodion surface, which latter has a filtering area of 250 sq. cm.

The apparatus is washed through by way of the serum reservoirs, first with 60 per cent alcohol, then with double distilled H_2O, and lastly with sterile 0.9 per cent NaCl solution. With the stop-cocks open, serum is now poured into one of the reservoirs, filling the space between the collodion tube and the glass tube *A* and mounting into the other reservoir. When both have been half-filled in this way, *a′* is connected with a pair of short vertical cylinders, *E* and *D*, containing mercury and joined by rubber tubing. The glass connection leading away from *a′* is sterile and is provided with a bulb packed with cotton to filter the air. Vertical excursion of the mercury columns in the cylinder *D* is provided for by attaching *E* to the circumference of a 10-inch wheel making 2 revolutions per minute, and the amount of the excursion is controlled by a screw-clamp, *G*. By this means pressure is brought to bear alternately on the serum in the reservoir *a′*, forcing it down into the space between glass and collodion or sucking it up again. The excess displacement is taken care of by the reservoir *b′* which is connected with a sterile rubber bag to prevent free entry of the atmospheric air while permitting change of the serum level. The result is that the fluid in contact with the collodion surface is constantly changed. If now the pressure in the filter flask *F* is diminished to 400 mm. Hg by means of the laboratory vacuum, filtration proceeds rapidly,—at a rate of about 4 cc. per minute to begin with, and somewhat more slowly as concentration progresses. The entire operation is carried out in an ice box at 2.5°C. In this way several hundred cubic centimeters of serum can be concentrated to about a fifth of its bulk within a few hours. The ultrafiltrate is sterile, colorless, protein-free and has the same freezing point as the original serum. The concentrate is



TEXT-FIG. 1

brownish red,—the ruddy tint being referable, as the spectroscope shows, to hemolysis imperceptible in the original serum,—but clear and not at all syrupy. The close agreement of the freezing points of ultrafiltrate and original serum, as well as the bulk of the separated fractions, attests the absence of any significant evaporation. This could be eliminated entirely by introducing into the flask a layer of sterile, washed paraffin oil, through which the ultrafiltrate would sink.

For determination of the protein concentrations in plasma and concentrate, Van Slyke's method was employed as has already been mentioned. Amounts of protein up to twice that contained in the animal's own total plasma were injected at body temperature into an ear vein of the rabbit used in the experiments. A test was made beforehand to find whether the concentrate hemolyzed or agglutinated the prospective recipient's blood. Hemolysis was never met and agglutination but rarely. In the latter case a compatible recipient was chosen. Since the serum had been outside the body more than 24 hours during the concentration there was no reason to expect the untoward effects recorded by Freund (8) for fresh serum; nor were they encountered. The injection was always well tolerated and the blood pressure remained high.

Hematocrit specimens taken after the injection of concentrate showed a diminution in the percentage of red cells much more considerable than could be accounted for by the fluid bulk introduced. It was plain that the increase in circulating protein had caused fluid to enter the blood from the tissues. The dye injection was not made until the blood volume had become approximately stationary. Hematocrit readings at this time indicated that the increase in volume was not great enough to reduce the protein percentage to that existing prior to the injection, and blood specimens taken just prior to dye injection proved that the concentration of protein per cc. of blood was indeed much increased, by as much as 40 per cent in individual instances, as already stated.

SPECIMEN PROTOCOLS

Rabbit Injected with Normal Plasma.—A male rabbit weighing 1200 gm. was fasted for 24 hours but allowed water. At 2:15 p.m. 0.7 cc. of 10 per cent amytal was given intravenously. Prompt hypnosis occurred, with a somewhat depressed respiratory rate. At 2:35 a first blood specimen was taken from the ear and at 2:50 the carotid was cannulated and the blood pressure tracing started. At 3:15, with the blood pressure at 70 mm. of Hg the injection of warmed heparinized plasma was begun. The first 20 cc. was given rapidly (in 2½ minutes) and the pulse rate slowed considerably, while the blood pressure rose. The injection was continued at a slower rate until 62 cc. in all had been introduced in 10 minutes. The blood pressure was now 80 mm. Hg. The animal was covered to conserve warmth and blood specimens were taken at intervals. The table shows the changes.

Time	Red cells	Serum protein	Blood pressure
p.m.	*per cent*	*gm. per cent*	*mm. Hg*
2:35	35	5.58	70
3:15	(62 cc. of plasma injected between 3:15 and 3:27)		
3:30	21	7.12	80
3:45	25		75
4:00	26		
4:30	27		73
5:00	27		
5:30	27		
6:00	27		65
6:30	26		Clotting in cannula
7:00	26		
7:30	26	7.58	

Rabbit Injected with Serum Concentrate.—A female rabbit weighing 1520 gm. was fasted for 24 hours. The red cell percentage was 43 and the serum protein 6.3 gm. per cent. At 1:40 p.m. 0.5 cc. of 10 per cent amytal was given intravenously. At 2:08 p.m. the carotid was cannulated; and at 2:21 the injection of warmed concentrated serum (24.7 gm. per cent protein) was begun. 12 cc. was injected in 4 minutes. An hematocrit reading taken 6 minutes later showed a red cell percentage of 34. At 2:50 the reading was 33, and at 3:00 again 34. The serum protein was now 8.4 gm. per cent.

In the animals of the foregoing protocols and in others treated in the same way, no dye was given. They were utilized merely to determine the course of the readjustment.

In the succeeding experiments dye was given at a time when the successive hematocrit readings indicated that the blood volume had become stationary. The amount of Chicago blue put into circulation was the same per cc. of plasma that yielded in normal rabbits a well marked coloration having the pattern indicative of the gradient of vascular permeability. Since staining intensity is conditional upon the concentration of dye in the plasma, allowance had to be made for the increase in plasma volume. This was calculated from the change in the red cell percentage, assuming that the original blood volume amounted to 5.5 per cent of the body weight. We were interested, not in the eventual distribution of dye throughout the tissue of the rabbit, but only its first escape from the blood. Consequently the fact that the amount of stainable tissue was unaltered by the procedure could be disregarded.

The staining encountered in animals treated as described and killed after the routine period of 10 minutes from the beginning of the injection showed significant and constant deviations from the normal. The coloration was less intense than in normal rabbits and more narrowly localized to the furthest capillary region—the region of greatest permeability. One may liken the picture to that of the staining in a normal rabbit with one wash of color removed.

In the experiments just described conditions had become stabilized, the readjustment of the blood bulk having ceased. In the following experiments dye was given together with the concentrate and its escape occurred while the readjustment was going on. The change in the percentage of circulating protein could not be determined directly in these cases because the blood carried a nitrogen-containing dye.

A male rabbit weighing 1860 gm. was given amytal at 2:15 p.m. By 3:01 p.m. the carotid artery had been cannulated and connected with the manometer. At 3:10, 4.5 cc. of an isotonic watery solution of Chicago blue and 2.5 cc. of concentrate were injected in 4 minutes and 40 seconds. After another 20 seconds a further injection of concentrate alone was begun and 8 cc. more of it was introduced in 8 minutes and 30 seconds. The skin vessels showed moderate dilatation as the injection proceeded. The carotids were cut 2 minutes after it ended. Autopsy showed the familiar, barred, blue pattern in the voluntary muscles with the bands somewhat narrower and of a lighter hue than under ordinary circumstances.

As control a 1900 gm. rabbit was anesthetized lightly with ether and given 3.7 cc. of the same dye solution in 3 minutes. The carotids were cut after exactly the same lapse of time as in the experiment just described. Autopsy showed the usual, relatively pronounced staining.

In Table I the blood changes in the animals of the two groups of experiments are summarized.

Effects of Hypertonic Solutions

To supplement the observations on the effects of increasing the plasma proteins, several experiments were made upon the course of the staining as influenced by the injection of a hypertonic solution of glu-

TABLE I

The Blood Changes in Animals with Increased Plasma Proteins

Rabbit		Body weight	Plasma volume	Amount of plasma or concentrate injected	Initial red cell percentage	Expected red cell percentage after injection	Actual red cell percentage stabilized	Elapsed period		Expected change in blood volume	Actual change in blood volume	Initial plasma protein concentration	Initial plasma protein	Amount of protein injected	Expected plasma protein concentration	Actual plasma protein concentration after injection	Elapsed period		Percentage change in protein concentration
		A. Animals in which the blood bulk had become readjusted before dye was given																	
		gm.	cc.	cc.	per cent	per cent	per cent	hrs.,	min.	per cent	per cent	gm. per cent	gm.	gm.	gm. per cent	gm. per cent	hrs.,	min.	per cent
Injected with plasma	P_1	1300	39	65	45	23	33	4	5	+96	+36	7.7	3.0			7.9	4	5	+3
	P_2	1200	42	62	36	19	26	4	5	+89	+38	5.58	2.3			7.58	4	5	+36
	P_3	1440	51	72	36	19				+89		6.73	3.4	4.7	6.6	7.74	4	50	+15
	P_4	1270	47	64	33	17	33	4		+94	0	6.6	3.1	4.2	6.6	7.2	4	55	+9
Injected with serum concentrate	B	1520	48	12	43	38	34		35	+13	+26	6.25	3.0	3.0	10.0	8.38		30	+34
	C	1740	59	15	38	33	28		30	+15	+36	6.14	3.6	4.2	10.5	7.28		30	+19
		B. Animals in which readjustment was taking place																	
	A	1860	64	11	37	33	29		3	+12	+28			2.8					
	D	1600	53	25	40	31	28		2	+29	+43			6.1					
	E	1680	52	14	44	38	34		3	+16	+29			3.5					

cose. The injection was intermitted while the dye was placed in circulation but was then resumed and continued until sacrifice of the animal. The fact is well attested that the readjustment of the fluid relationships is exceedingly rapid after the introduction of non-isotonic solutions of crystalloids. In order to ensure a continuous passage of fluid from the tissues to the blood while the dye was distributing itself, the glucose solution was continuously but slowly injected during the entire period from immediately after the dye injection until the animal was killed.

Specimen Protocol.—A rabbit weighing 1675 gm. was fasted for 24 hours. At 2:25 p.m. the red cell percentage was 44. At 2:32, 1 cc. of 10 per cent amytal was given intravenously; and at 3:15 the carotid was cannulated. The blood pressure was 90 mm. of Hg. At 3:32, 2 cc. of 70 per cent glucose was given in 60 seconds into an ear vein. At once thereafter injection of the calculated dose of Chicago blue was begun, and when this was completed, after 2½ minutes, the glucose injection was resumed and continued for 7½ minutes. By that time 11 cc. of 70 per cent glucose had been injected in all. The animal was now exsanguinated by cutting the carotids. The red cell percentage as obtained on the carotid blood was now 36, indicating a 22 per cent increase in the blood volume and a 40 per cent increase in the plasma volume. At autopsy the muscles showed the pattern characteristic of the gradient of vascular permeability, the findings not differing noticeably from the normal. The skin was deeply colored and there was no observable dryness of the tissues. The bladder was full and there was a small amount of dye in the urine.

Despite the great increase in the plasma volume of this animal, an increase which involved, of course, a passage of fluid from the tissues to the blood, as well as a dilution of the dye in the blood, the staining of the muscles and skin did not differ from the normal either in intensity or in distribution. The skin is known to supply water to the blood in quantity when hypertonic solutions are given, and Adolph and Lepore's recent work indicates that the muscles yield some of it. The reason for the difference in the findings when the blood volume has been increased with plasma and with glucose respectively is dealt with further on. For the present it will suffice to point out that the movement of fluid from the tissues to the blood does not essentially alter the gradient of vascular permeability or check the process of staining. The findings yield proof in addition to that of previous work (2) that the spread of dye from the blood to the tissues cannot be essentially dependent upon fluid movement, as some have supposed.

Effects of Reducing the Plasma Proteins

The percentage of circulating plasma proteins can be readily reduced by plasmapheresis. As ordinarily carried out this involves the repeated return of washed red cells suspended in Locke's solution. There is much in the literature to suggest that the best of salt solutions may harm the capillary endothelium, and it is essential to prevent such injury if one is to measure capillary permeability. In an initial series of experiments recourse was had to the ability of the animal to make up its own blood bulk after each removal of cells. Robertson and Bock have shown that forcing water by the alimentary tract after hemorrhage results in a remarkably rapid and effective restoration of the blood volume (9). Utilization was made of this phenomenon.

Warmed water or Ringer's solution was administered to amytalized rabbits by stomach tube, to the amount of 120 cc., and soon afterwards the anesthetized animal was bled repeatedly at short intervals and the cell sediment obtained on centrifugation was returned to the body by way of a femoral vein. To replace the loss of the first bleeding the cells from a compatible donor were used. Thereafter those of the host were injected, as obtained at each previous bleeding. The bleedings were carried out rapidly from a carotid artery into a 1 per cent heparin solution (1 cc. to 30 cc. of blood). The mixture was centrifuged and the plasma pipetted off as completely as possible. The cells were then suspended in 0.9 per cent NaCl, recentrifuged, the fluid discarded, and the sediment filtered through gauze before injection to remove the small amount of white granular fibrin which was always present in spite of the heparin. It was found that by the repeated bleedings and reinjections the plasma proteins could be reduced by about one-third in the course of 3 hours. The blood pressure tended to fall gradually but there was no other evidence of circulatory disturbance. The blood volume underwent a slight decrease as indicated by changes in the proportion of red cells in hematocrit specimens.

The amount of dye to be introduced was calculated on the basis of the plasma volume existing at the time of the injection. The blood pressure tracing was continued until the animal was killed.

In some further experiments the washed cells were made up to the original volume by the addition of ultrafiltrate obtained as a by-product to the concentration of protein by the method already described. Although the plasma was thus replaced by a fluid which lacked only the proteins of the blood, the blood pressure and volume fell to approximately the same extent as when no addition had been made to the cells prior to their reinjection.

Incidentally to the work, a number of tests were made upon rabbits of the merits of ultrafiltrate and of Ringer-Locke solution respectively, in restoring blood volume after hemorrhage. The carotid tracings did not indicate that the ultrafiltrate had any special advantages.

Chicago blue 6B, given when the blood protein had been much reduced by plasmapheresis and the blood volume had become stationary, yielded a staining of the muscles notably greater than normal. This happened although the systemic blood pressure had fallen somewhat as a result of the lessening in blood bulk. The bars indicative of the gradient of permeability were broader and more intense than ordinary, and in addition to them there was some generalized staining. In summary of the findings, one can say that the staining was like that in a vitally stained normal rabbit but with an extra wash of color everywhere superimposed.

TABLE II

The Blood Changes in Animals with Decreased Plasma Proteins

Rabbit	Body weight	Plasma volume	Amount of plasma removed	Elapsed time	Initial red cell percentage	Final red cell percentage	Elapsed time after last removal of plasma	Percentage change in blood volume	Initial plasma protein concentration	Initial plasma protein	Approximate amount of protein removed	Final plasma protein concentration	Elapsed time after last removal of plasma	Percentage change in protein concentration
	gm.	*cc.*	*cc.*	*hrs., min.*	*per cent*	*per cent*	*hrs., min.*	*per cent*	*gm. per cent*	*gm.*	*gm.*	*gm. per cent*	*hrs., min.*	*per cent*
L	1750	62	67	2 30	36	43	17	−16	6.36	4.0	3.4	3.79	17	−42
M	1920	65	97	3	38				6.93	4.5	5.0	3.37	13	−51
N	1500	43	42	1 45	48	39	30	+23	7.48	3.2	2.9	6.4	30	−14
O	1900	65	60	2 37	38	39	1 20	−2	6.24	2.7	2.9	3.35	1 20	−46
P	1830	58	56	2 42	42	43	1 42	−2	5.65	3.3	2.6	3.63	1 42	−36
Q	1830	62	54	3	38	39	2 55	−2	6.11	3.8	2.8	3.58	2 55	−41
R	1850	61	60	3	40	43	2 45	−7	5.82	3.4	2.9	3.76	2 45	−35
X	2300	80	75	2	37	40	20	−7	6.13	4.8	3.5	3.16	20	−48
T	2000	74	74	3	39	40	1 10	−2	5.8	3.9	3.3	3.18	1 10	−45
U	1850	61	105	3 24	40	46	10	−13	5.82	3.6	4.4	2.63	10	−55
V	1980	64	78	1 45	41	43	2	−5	5.8	3.7				
W	1900	67	74	1 40	36	48	1 10	−25	6.13		3.0	4.04	3	−34

Table II presents the blood findings in the animals treated as just described.

The Influence of Adsorption of the Dyes

The experiments prove that Chicago blue escapes more readily from the blood when its plasma proteins have been reduced, and with greater difficulty when they have been increased. One cannot conclude forthwith, however, that the differences are the result of a lessened or increased colloid osmotic pressure; for the possibility exists that they are due, in part at least, to adsorption of the dye on the plasma proteins. Marshall and Vickers have found that ultrafiltration of an aqueous solution of phenolsulfonphthalein through a collodion membrane yields a fluid of the same dye concentration as the original solution, but that ultrafiltration of a plasma mixture yields a more dilute filtrate (10). They suggest that certain phenomena in the renal elimination of phenolsulfonphthalein may be due to adsorption of the dye on the plasma proteins. Grollman showed that phenol red is indeed adsorbed on the blood proteins, more especially on the albumin fraction (11).

Tests for the fixation of Chicago blue on blood proteins by the method of ultrafiltration through collodion membranes are rendered difficult by the indiffusibility of the dye. Even through highly permeable membranes little passes from an aqueous solution in the course of several days. However, the diffusion method of Northrop and Anson in which a partition of porous glass is utilized (12), proved admirable for our purpose.

The cell, sealed with the porous glass, was filled with fluid containing a known amount of dye, and the stop-cock was closed, thus preventing alterations in the volume of the fluid by osmotic transport of water, which might have interfered with the free diffusion of the dye. An ultrafiltrate of rabbit serum, prepared in the way already described, was run into the outer jacket in sufficient quantity just to cover the porous disc. The fluids had been cooled previously to 2.5°C. and the apparatus was kept at this temperature.

The ultrafiltrate outside of the cell was drawn off and replaced at regular intervals of time and the amount of dye that had passed into it was estimated colorimetrically. This amount became constant when the concentration gradient of dye in the membrane had established itself, and this in turn was a function of the diffusibility of the dye and of the concentration within the cell of its freely diffusible portion, as distinct from that adsorbed on the blood proteins. The porous disc employed was 0.5 mm. thick and impermeable to proteins within the time limit of our experiments, as proven by preliminary tests specifically directed to the point.

Equimolecular solutions of Chicago blue in ultrafiltrate and in rabbit serum were employed successively within the cell, and possible changes in the permeability of the membrane resulting from adsorption upon it of protein were controlled by a second series of determinations with dye in ultrafiltrate, carried out immediately after the cell had been emptied of serum, without washing it. In all instances the dye was present in the amount per cc. that had been introduced into the plasma of the rabbits used in our experiments.

The colorimetric determinations showed that the dye diffused more than three times as slowly from serum as from ultrafiltrate. Consequently the specimens of diffusate were removed every hour in the case of serum, and every 15 minutes in the case of the ultrafiltrate. Each specimen, as obtained, was made up to 25 cc. with Ringer's solution and compared in a Duboscq colorimeter with a 1:16,000 dilution of Chicago blue in Ringer's solution. During the readings the column of diffusate was kept constant (at 35) and that of the standard was varied. It follows that the figures procured are directly indicative of the relative concentration of dye in the various specimens. Thus for example the concentration of dye in a first specimen of ultrafiltrate, as recorded in the type instance of Table III, was nearly twice as great as in the last of the same series (25 as compared with 14).

TABLE III

Influence of Plasma Proteins on the Diffusion of Chicago Blue 6B

	Dye in ultrafiltrate (15 min. specimens)	Dye in serum (hr. specimens)	Dye in ultrafiltrate (15 min. specimens)
Period of adjustment	25	41	37
	21		25
	18		21
	16		19.5
	15	32	16
			15
		25	
		21	
Period of diffusion at constant rate	14	19	14
	14		14
	14		14
	14	19	
		19	
Relative amounts of dye escaping per hr. during constant period	**56**	**19**	**56**

It will be seen from Table III that dye diffused with far greater rapidity from the filtrate than from the serum. Since the amount of dye passing in unit time through the disc after the concentration gradient becomes uniform can be taken as a measure of the freely diffusible dye, it would appear that, in the instance given, as in others not here recorded, only about 30 per cent of the Chicago blue in the serum mixtures was available for diffusion, the remainder being adsorbed on the plasma proteins. The objection that the pores of the disc had become clogged with protein particles is disposed of by the control observations made after the use of a serum-dye mixture. The figures indicate that there was no mechanical clogging; indeed more fluid came through into the first control specimen than into the corresponding one of the preliminary series.

By a test of another sort the adsorption of Chicago blue was readily demonstrated. A marked reduction of the tinctorial value of phenol red is associated with its adsorption upon the blood colloids. The same holds true of Chicago blue, dilute solutions in serum being less intensely colored than similar ones in Ringer's fluid. To test whether this holds true when the dye is present in the amount per cc. of plasma employed in our animals, it has been necessary to utilize thin layers of fluid, since the deep color of the solutions tends to mask differences between them. Small drops of serum and of salt solution containing dye in the same appropriate quantity were placed side by side in a hemocytometer chamber having a double dais. Comparison showed a pronounced difference in their intensity of color, the mixture with salt solution being by far the deeper blue. No attempt was made to quantitate the difference.

DISCUSSION

The experiments show that when the percentage of the blood proteins is experimentally increased, the passage of Chicago blue from the muscle capillaries into the tissue takes place more slowly than usual and is confined in the beginning to the furthest portion of these vessels. When the proteins are decreased the dye escape is more abundant than usual and extends further back along the capillaries. In both cases the staining pattern is still indicative of an increasing permeability of the vessel wall as the venule is neared.

Are the altered osmotic conditions resulting from increase or decrease of the blood colloids responsible for these changes? Assuming this one might suppose that the passage of water from the tissue to the blood, consequent upon a sudden experimental increase in the blood proteins, would involve a flow in the opposite direction to that of dye escape, hindering the latter. But as a matter of fact, when dye is injected together with a hypertonic solution of glucose, and water is rapidly drawn into the circulation while dye passes out into the tissues, one finds no alteration in the distribution of dye to muscle or skin. The injection of dye concurrently with a plasma concentrate rich in blood proteins yields a staining quite as good as when the circulation has become stabilized after introduction of the concentrate, although water is doubtless passing from the muscle into the blood (6) while the coloration is going on.

If the osmotic pressure of the blood proteins does not condition the escape of dye, what can be the cause for the alterations encountered when the protein percentage has been changed? Our tests indicate that the amount of blood protein upon which the dye can become adsorbed is the effectual factor. Chicago blue when thus adsorbed is largely removed from the possibility of diffusion, as shown by our *in vitro* experiments; and the rate of staining with a dye is conditional not only upon its diffusibility, but upon the available amount of it (13, 1). The intensity of the staining of muscle with Chicago blue, the effective extent of the gradient of capillary permeability, and the presence or absence of diffuse coloration along the capillaries in addition to the local staining referable to the gradient, all have been found to vary with the amount of dye in circulation. The effective amount, in our experiments involving alterations in the per cent of plasma proteins, varied with this percentage, more or less being available accordingly as the percentage of circulating blood protein was decreased or increased.

It might be urged that adsorption upon tissues must also be reckoned with as complicating the findings; but Chicago blue is not immediately adsorbed in perceptible amounts upon the tissue of muscles into which it escapes (2). One has then only to consider adsorption upon the blood proteins, and this will account for the observed differences in the amount of dye escaping from the blood. The most striking evidence of the influence of adsorption upon the proteins was obtained in the plasmapheresis experiments in which no fluid was added to the red cells restored to the circulation. In these experiments the carotid blood pressure fell, owing in part at least to a gradual lessening of the blood bulk. From previous knowledge it seems certain that there had been some compensatory restriction of the muscle circulation (14). Nevertheless the escape of dye from the capillaries was more abundant and the extent of the gradient of permeability was greater than under normal conditions, while, furthermore, dye escaped everywhere along the capillaries as does not happen ordinarily,—all this being due, it would appear, to the diminution in blood protein, which left more of the injected dye available for diffusion.

Can it be that the greater and greater escape of dye as the blood carries it along the capillary way, an increase essentially independent of the hydrostatic pressure (2) and of osmotic conditions, is caused by a progressive falling off in the amount of dye adsorbed upon the blood proteins as the blood flows along, with result that more becomes available progressively for escape through the capillary wall? Such a view entails the assumption that the changes taking place in the blood in its passage from arteriole to venule are such as would act to lessen adsorption. If this were the case, the gradient should vary directly with the magnitude of these changes, being less marked and even absent in proportion as they are prevented. Several facts negate this possibility. After section of the nerves to mammalian muscle the gradient persists although the blood undergoes but little alteration during its rapid passage through the dilated capillaries. The blood entering the capillaries of frog skin is essentially venous, having the lowest tension of oxygen and the highest of carbon dioxide to be found anywhere in the circulation, and it is rendered arterial on its way to the venules. Despite this reversal of the ordinary condition of affairs along capillaries, dyes escape in greater and greater amount from these vessels as the venules are neared, just as in the case of mammalian capillaries. Furthermore, when the circulation is cut off after dye has been allowed to enter the vessels of the mouse's ear, the staining that gradually ensues from capillaries dilated as the result of a previous ischemia shows that the gradient of dye distribution still exists, although now the stagnant blood has become venous everywhere. These facts would seem to rule out the possibility that the progressive release of adsorbed dye is responsible for the increasing escape along the capillaries.

The fact that the gradient of capillary permeability exists independently of alterations in the percentage of the blood proteins is, of course, far from meaning that these are devoid of effect upon exchange between the blood and tissues. The edema of nephrosis and that produced experimentally by plasmapheresis (15) attest to an important influence of the proteins to hold water in the circulation as do some of the phenomena of the present experiments, notably the persisting increase in blood bulk after the injection of serum concentrate.

In theoretical considerations of the influence of osmotic pressure upon exchange through the capillary wall, the conditions have too often been oversimplified. The capillary is far from being completely impermeable to protein (16) and it lets through much more albumin than it does globulin. Osmotic pressure must always be referred to the particular membrane with which it is measured, for the osmotic forces of only those elements to which the membrane is impermeable are effective. If the collodion membrane utilized in osmotic pressure determinations held back all electrolytes, instead of the protein only, serum would give a value of 7 atmospheres instead of 40 mm. of Hg. As it is, collodion membranes impermeable to all the proteins are employed in tests for the osmotic pressure of serum, not membranes which let albumin through with relative ease; and these tests are made against an ultrafiltrate devoid of protein, not one containing it as lymph actually does. In consequence figures are obtained considerably higher than those to be expected had a capillary wall been utilized. The fact that this wall is not everywhere equally permeable, but becomes progressively more so as the venule is neared, complicates the state of affairs.

It is pertinent to inquire whether adsorption upon plasma proteins may not condition the distribution to the tissues of some normal substances as it does that of our dyes. That a large proportion of the bilirubin circulating in jaundice cases is adsorbed upon the proteins has recently been recognized (17).

In previous work the fact has been brought out that the escape of vital dye in progressively increasing amount along the capillaries is a phenomenon that occurs independently of alterations in hydrostatic pressure, though its effective extent is influenced thereby. The same has now been found to hold true as concerns alterations in the percentage of plasma proteins. One must conclude, as from other evidence, that the gradient of vascular permeability disclosed by the escape of dye cannot be due to an interplay of hydrostatic and osmotic forces such as has generally been held to control exchange between the blood and tissues. Whatever the nature of the gradient,—and the evidence points to a structural cause for it,—it is a dominating factor in exchange.

SUMMARY

The influence of osmotic conditions on the gradient of capillary permeability disclosed by the distribution into mammalian muscle of vital dyes has been tested experimentally. The percentage of circulating blood proteins was increased in rabbits by the injection of compatible plasma, or of compatible serum concentrate obtained by means of a new method of ultrafiltration which has proved both rapid and effective. It was found that when this had been done and the circulatory conditions had stabilized themselves, the gradient of capillary permeability still existed, though its effective extent was less than under normal circumstances. When the percentage of circulating blood proteins was reduced, on the other hand, by repeated bleedings with return of the cells, either as such or suspended in the protein-free fluid obtained by dialysis of serum, the extent of the gradient of capillary permeability was broadened and dye passed out into the tissue more readily than usual from the capillary as a whole. In contrast to these findings injection of a very hypertonic dextrose solution during the period when dye was escaping had no perceptible effect on the gradient of capillary permeability.

The observed phenomena cannot be explained by a flow of dye-stained fluid into or out of the blood vessels. The gradient of capillary permeability exists independently of osmotic conditions, though its extent can be markedly influenced by altering the amount of circulating blood proteins. A considerable proportion of the dye used to study the gradient is adsorbed upon these proteins, as subsidiary experiments have shown. This happening provides a sufficient cause for the differences observed in the extent of the gradient when the percentage of proteins is increased or diminished.

The evidence like that of previous papers indicates that the cause for the gradient is to be found in a structural differentiation along the capillary, such that the barrier offered by its wall progressively diminishes on the way to the venule. Most current estimates of the effective osmotic pressure of the blood proteins fail to take into account the existence of local differences in permeability along the capillary.

BIBLIOGRAPHY

1. Rous, P., Gilding, H. P., and Smith, F., *J. Exp. Med.*, 1930, **51**, 807.
2. McMaster, P. D., Hudack, S., and Rous, P., *J. Exp. Med.*, 1932, **55**, 203.
3. Hamburger, H. J., Osmotische Druck und Ionenlehre in ihrer Bedeutung fur die Physiologie und die Pathologie des Blutes, Berlin, Allgemeine Medizinsche Verlagsanstalt, 1912.
4. Boycott, A. E., and Douglas, C. G., *J. Path. and Bact.*, 1909, **13**, 256.
5. Govaerts, P., *Compt. rend. Soc. biol.*, 1925, **93**, 441.
6. Adolph, E. F., and Lepore, M. J., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 963.
7. Brown, W., *Biochem. J.*, 1915, **9**, 591.
8. Freund, H., *Deutsch. klin. Woch.*, 1926, **2**, 2187.
9. Robertson, O. H., and Bock, A. V., Reports of the Special Investigation Committee on Surgical Shock and Allied Conditions, No. 6, Memorandum on blood volume after haemorrhage, 1918.
10. Marshall, E. K., Jr., and Vickers, J. L., *Bull. Johns Hopkins Hosp.*, 1923, **34**, 1.
11. Grollman, A., *J. Biol. Chem.*, 1925, **64**, 141.
12. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928–29, **12**, 543.
13. Schulemann, W., *Biochem. Z.*, 1917, **80**, 1.
14. Rous, P., and Gilding, H. P., *J. Exp. Med.*, 1929, **50**, 189.
15. Leiter, L., *Proc. Soc. Exp. Biol. and Med.*, 1928, **26**, 173.
16. Krogh, A., The anatomy and physiology of the capillaries, New Haven, Yale University Press, revised and enlarged edition, 1929.
17. Barron, E. S. G., *Medicine*, 1931, **10**, 77.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1932,
Vol. 56, No. 3, pp. 399-409]

SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS (PRECIPITIN REACTIONS)

BY K. LANDSTEINER, M.D., AND J. VAN DER SCHEER

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, May 23, 1932)

Serological reactions with chemical compounds of simple constitution were demonstrated for the first time by means of the so called inhibition reaction (1, 2). This subject has been discussed (1), and the arguments given for the view that the inhibition phenomena are caused by a combination of the inhibiting substances with antibodies, thereby preventing their precipitating action. Although there is no reasonable doubt as to the validity of this explanation which is strongly supported by the close similarity in the specificity of inhibition and precipitin reactions and the analogy to the inhibition produced by an excess of precipitable substances, it has remained of value to furnish direct experimental proof of the combination of antibodies with low molecular compounds by a reaction which does not involve the use of protein antigens.

In experiments on anaphylaxis to azoproteins it was shown that previous injection with azodyes protected sensitized guinea pigs against the shocking action of azoproteins containing the same azo component (3–5). Since this protection was still evident after the dyes had practically disappeared from the circulation, the effect was attributed to a neutralization of antibodies. In some cases the injection of the dyes was even followed by typical anaphylactic symptoms. Klopstock and Selter (6) reported that they obtained complement fixation reactions with immune sera to azoproteins, and emulsions of lecithin preparations to which had been added diazotized *p*-arsanilic acid or metanilic acid. Interesting experiments were carried out by Marrack and Smith (7) who were able to show that the diffusion of an azodye prepared from *p*-arsanilic acid is interfered with by the addition of an immune serum specific for the azo component.

Attempts made in the course of studies on azoproteins to produce specific precipitin reactions with simple azodyes did not lead to consistent results. Recently, a special group of azodyes was found with which such reactions could easily be demonstrated. These substances were made from a series of aminoanilic acids, with aliphatic side chains containing from two to eight carbon atoms, by diazotization and coupling to resorcinol or tyrosine. For the production of the immune sera, antigens were prepared by diazotizing the same aminoanilic acids and coupling the diazo compounds to protein.

Technique

Preparation of Nitroanilic Acids.—The method used was essentially the same as that described in a previous paper (8).[1] A finely ground mixture of equimolecular quantities of dicarboxylic acids (malonic, succinic, glutaric, adipic, pimelic, and suberic acids), and para-nitroaniline was melted in a paraffin bath at 170–175°C., and the mixture was stirred continuously for a period of 45 minutes. In the preparation of nitromalonic acid, the temperature of the bath was kept at only 130–135°C. In all cases except that of succinic acid it was found necessary to use fused anhydrous zinc chloride in the condensation in order to obtain satisfactory yields.

The powdered zinc chloride (8 gm. for 13.8 gm. of *p*-nitroaniline) was added to the melt in 5 portions during the first 15 minutes. After heating for 45 minutes, the mass was poured into about 10 volumes of water in a porcelain dish and heated on the steam bath until broken up. During this process, concentrated sodium hydroxide was added in small amounts until the solution remained alkaline to litmus. After filtration, the insoluble material was finely ground and extracted a second time with hot water and alkali in the same manner. The aqueous extracts were combined, made neutral to litmus, and after standing in the ice box overnight, the filtered solution was made acid to Congo red by addition of concentrated hydrochloric acid The precipitate was washed with water and dried. Of para-nitromalonanilic acid only 20 per cent of the theoretical yield was obtained due to decomposition of malonic acid during the fusion. In the other cases the yield was 40 to 50 per cent.

The following nitroanilic acids were prepared.

Para-nitromalonanilic acid (NO_2–C_6H_4–NH–CO–CH_2–COOH): Crystallized from boiling 25 per cent alcohol. Microscopic platelets. Melting point: 157°C., with gas formation.

Titration: 0.1279 gm., dissolved in 80 per cent alcohol, required for neutralization 5.7 cc. N/10 NaOH. Formula $C_9H_8O_5N_2$ requires 5.7 cc.

Para-nitrosuccinanilic acid (NO_2–C_6H_4–NH–CO–CH_2–CH_2–COOH): Crystallized from 25 parts of boiling 25 per cent alcohol. Long, microscopic, prismatic platelets. Melting point: 194–195°C.

---

[1] *p*-Nitrooxanilic acid was made according to the method described by Ossian Aschan (9).

Titration: 0.119 gm., dissolved in 80 per cent alcohol, required for neutralization 5 cc. N/10 NaOH. Formula $C_{10}H_{10}O_5N_2$ requires 4.96 cc.

Para-nitroglutaranilic acid ($NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-COOH$): Crystallized from 40 parts of boiling 25 per cent alcohol. Long, thin, microscopic crystals. Melting point: 170–171°C.

Titration: 0.2199 gm., dissolved in 80 per cent alcohol, required for neutralization 8.7 cc. N/10 NaOH. Formula $C_{11}H_{12}O_5N_2$ requires 8.72 cc.

Para-nitroadipanilic acid ($NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-COOH$): Crystallized from 25 parts of boiling 50 per cent alcohol. Narrow microscopic platelets. Melting point: 174–175°C.

Titration: 0.133 gm., dissolved in 80 per cent alcohol, required for neutralization 5 cc. N/10 NaOH. Formula $C_{12}H_{14}O_5N_2$ requires 5 cc.

Para-nitropimelanilic acid ($NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$): Crystallized from 50 parts of boiling 25 per cent alcohol. Long, narrow, microscopic platelets. Melting point: 147–148°C.

Titration: 0.1530 gm., dissolved in 80 per cent alcohol, required for neutralization 5.45 cc. N/10 NaOH. Formula $C_{13}H_{16}O_5N_2$ requires 5.46 cc.

Para-nitrosuberanilic acid ($NO_2-C_6H_4-NH-CO-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-COOH$): The crude substance often contained some suberic acid from which it was easily freed by redissolving in water with sodium hydroxide and partial precipitation from this solution with hydrochloric acid which caused the nitro compound to separate first. It was then dissolved in boiling 50 per cent alcohol and crystallized upon cooling of the hot solution as microscopic platelets. Melting point: 158–159°C.

Titration: 0.147 gm., dissolved in 80 per cent alcohol, required for neutralization 5.05 cc. N/10 NaOH. Formula $C_{14}H_{18}O_5N_2$ requires 5 cc.

Preparation of Aminoanilic Acids.—The nitroanilic acid, dissolved in about 3 parts of water by addition of a slight excess of ammonium hydroxide and heating, if necessary, was added to a hot solution of ferrous sulfate, 7 aq. (6.5 mols for each mol of nitroanilic acid) in 2.5 parts of water. A 28 per cent ammonia solution (10 cc. for each 12 gm. of ferrous sulfate, 7 aq.) was added in 5 equal portions over a period of 10 minutes, shaking well with each addition. After 15 minutes heating on the steam bath, the ferric hydroxide was removed by filtration and to the clear filtrate enough 10 per cent hydrochloric acid was added to obtain maximum precipitation of the amino compound. The precipitate was filtered off on a Buchner funnel after standing in the ice box overnight. It was freed from a very small amount of acid-insoluble material by redissolving in a small amount of water with a slight excess of dilute hydrochloric acid and reprecipitating from the filtrate by addition of the required amount of dilute sodium hydroxide. After standing in the ice box overnight, the substance was filtered off and dried *in vacuo* at 50° over calcium chloride. 70 to 80 per cent of the theoretical yield was obtained.

Para-aminomalonanilic acid ($NH_2-C_6H_4-NH-CO-CH_2-COOH$): Crystallized from hot water using norit for decolorizing. White microscopic platelets. Melting point: 175–176°C., with gas formation.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_9H_{10}O_3N_2$ calculated N 14.43 per cent, found 14.31 per cent.

Para-aminosuccinanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–COOH): Crystallized from 15 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 183–184°C., with darkening.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{10}H_{12}O_3N_2$ calculated N 13.46 per cent, found 13.30 per cent.

Para-aminoglutaranilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–COOH): Crystallized from boiling water, using norit for decolorizing. Microscopic platelets. Melting point: 186–187°C., with darkening.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{11}H_{14}O_3N_2$ calculated N 12.61 per cent, found 12.20 per cent.

Para-aminoadipanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from 15 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 165–166°C.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{12}H_{16}O_3N_2$ calculated N 11.86 per cent, found 11.57 per cent.

Para-aminopimelanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 178–179°C., with darkening.

Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{13}H_{18}O_3N_2$ calculated N 11.20 per cent, found 11.06 per cent.

Para-aminosuberanilic acid (NH_2–C_6H_4–NH–CO–CH_2–CH_2–CH_2–CH_2–CH_2–CH_2–COOH): Crystallized from 100 parts of boiling water, using norit for decolorizing. Long, narrow, microscopic platelets. Melting point: 162–163°C.

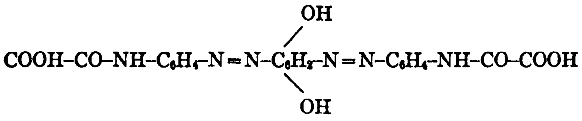
Kjeldahl determination after drying at 100° *in vacuo* over sulfuric acid: $C_{14}H_{20}O_3N_2$ calculated N 10.61 per cent, found 10.38 per cent.

Para-aminooxanilic acid (NH_2–C_6H_4–NH–CO–COOH): Prepared by condensation of para-phenylenediamine and oxalic acid following the methods described by G. Koller (10) and by W. A. Jacobs and M. Heidelberger (11). The product thus obtained was redissolved in about 30 parts of water with a slight excess of dilute sodium hydroxide. After making neutral to litmus, the solution was heated and decolorized with norit. The colorless filtrate was made faintly acid to Congo by addition of hydrochloric acid and the precipitated para-aminooxanilic acid was filtered and dried *in vacuo* at 50°.

Preparation of Azodyes.—Resorcinoldisazo-*p*-anilic acids: 2 millimols of aminoanilic acid were dissolved in 50 cc. of water and 6 cc. of normal hydrochloric acid and diazotized at 0–5°C. by slow addition of 2 cc. of normal sodium nitrite. After the diazotization was complete (test with starch iodide paper), 1 millimol of resorcinol (110 mg.) dissolved in 40 cc. N/2 sodium carbonate solution, cooled to 0–5°, was rapidly added with stirring, and the mixture was kept at 0–5° for ½ hour. Enough dilute hydrochloric acid was added to make the solution weakly acid to Congo and bring about complete precipitation of the dye, which was then separated from the liquid by centrifugalization. After washing twice in the centrifuge tube with 50 cc. of water, the dye was redissolved in 40 cc. of water by addition of a slight excess of normal sodium hydroxide and was reprecipitated by the addition of hydrochloric acid. It was again washed three times with 50 cc. of water and dried at 75° *in vacuo* over calcium chloride. The yield was from 97 to 99 per cent of the theory.

The following values for nitrogen found by analysis are calculated for ash-free substance. The content of ash in the dyes varied between 1 and 2 per cent with the exception of the resorcinoldisazo-*p*-oxanilic acid which contained 6.2 per cent ash.

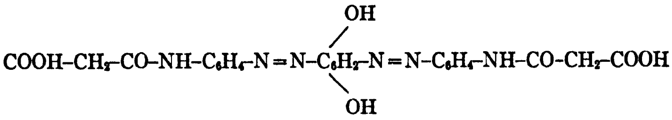
Resorcinoldisazo-*p*-oxanilic acid:



$$\mathrm{COOH{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\underset{\displaystyle \diagdown OH}{\overset{\displaystyle OH \atop \diagup}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}COOH}$$

Calculated N 17.01 per cent, found 16.89 per cent.

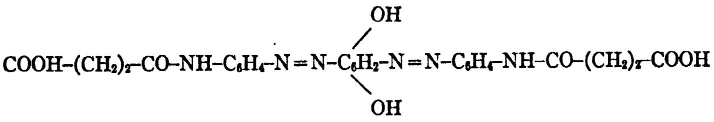
Resorcinoldisazo-*p*-malonanilic acid:



$$\mathrm{COOH{-}CH_2{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\underset{\displaystyle \diagdown OH}{\overset{\displaystyle OH \atop \diagup}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}CH_2{-}COOH}$$

Calculated N 16.16 per cent, found 16.01 per cent.

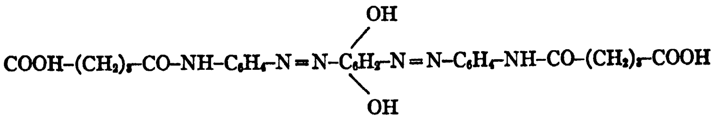
Resorcinoldisazo-*p*-succinanilic acid:



$$\mathrm{COOH{-}(CH_2)_2{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\underset{\displaystyle \diagdown OH}{\overset{\displaystyle OH \atop \diagup}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}(CH_2)_2{-}COOH}$$

Calculated N 15.33 per cent, found 15.56 per cent.

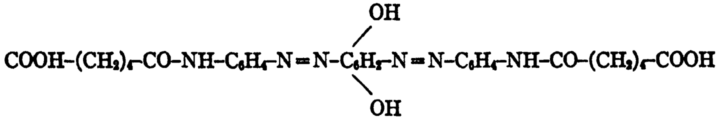
Resorcinoldisazo-*p*-glutaranilic acid:



$$\mathrm{COOH{-}(CH_2)_3{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\underset{\displaystyle \diagdown OH}{\overset{\displaystyle OH \atop \diagup}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}(CH_2)_3{-}COOH}$$

Calculated N 14.58 per cent, found 14.35 per cent.

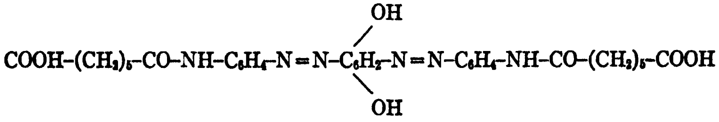
Resorcinoldisazo-*p*-adipanilic acid:



$$\mathrm{COOH{-}(CH_2)_4{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\overset{OH}{\underset{OH}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}(CH_2)_4{-}COOH}$$

Calculated N 13.91 per cent, found 14.03 per cent.

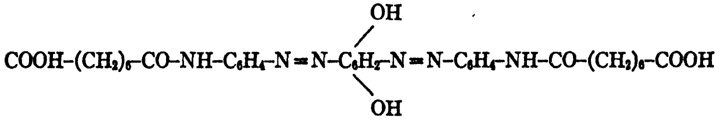
Resorcinoldisazo-*p*-pimelanilic acid:



$$\mathrm{COOH{-}(CH_2)_5{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\overset{OH}{\underset{OH}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}(CH_2)_5{-}COOH}$$

Calculated N 13.29 per cent, found 13.10 per cent.

Resorcinoldisazo-*p*-suberanilic acid:



$$\mathrm{COOH{-}(CH_2)_6{-}CO{-}NH{-}C_6H_4{-}N{=}N{-}\overset{OH}{\underset{OH}{C_6H_2}}{-}N{=}N{-}C_6H_4{-}NH{-}CO{-}(CH_2)_6{-}COOH}$$

Calculated N 12.73 per cent, found 12.81 per cent.

Preparation of Azoproteins.—1 millimol of aminoanilic acid was diazotized as described before at 0–5° and the diazo solution added to a cold solution containing 540 mg. of horse serum-globulin (prepared from diluted horse serum by half-saturation with ammonium sulfate and subsequent dialysis to remove the salt) and 13 cc. of normal sodium carbonate. The total volume of the mixture was approximately 65 cc. Coupling was allowed to proceed for ½ hour at 0–5° and the azoprotein was precipitated by addition of enough hydrochloric acid to make the solution weakly acid to Congo. The precipitate was separated from the liquid by centrifugalization and was washed several times with weakly acidulated saline. It was redissolved in saline by means of dilute sodium carbonate, making the solution neutral to litmus. As a preservative, 0.25 per cent phenol was added. The solutions used for the injections contained 5 mg. of azoprotein pro cc.

Immunization.—Four azoproteins were used for immunization; namely, those prepared from *p*-aminooxanilic acid, *p*-aminosuccinanilic acid, *p*-aminoadipanilic acid, and *p*-aminosuberanilic acid by coupling to horse serum-globulin as described above. For the immunization with each antigen four rabbits were used, each receiving daily intravenous injections of 2 cc. of the antigen solution for 6 days. One or two more courses of injections were given at intervals of 1 week,[2] and the sera were tested 7 days after the last injection. Two to three active sera were obtained in each series. For convenience the sera will be referred to as oxanilic, succinanilic, adipanilic, and suberanilic immune sera.

EXPERIMENTAL

Precipitin Reactions with Azodyes.—The immune sera obtained in the manner described, with the exception of the oxanilic immune sera, gave distinct precipitin reactions when added to solutions of the sodium salts of the corresponding resorcinol-azodyes (Table I).[3]

Results practically identical with those recorded in the table were obtained with the other sera at our disposal.

The reactions with the succinanilic sera were very specific whereas the action of the immune sera for the antigens with longer side chains extended to the compounds next in the series. The same sort of specificity appeared in tests with azoproteins. These observations will be described in a subsequent communication.

The strongest reactions occurred with the suberanilic immune sera and the homologous dye. Upon the addition of the antiserum, a distinct turbidity appeared almost immediately and after ½ hour small flakes, and later larger ones were formed, the phenomenon resembling in all respects a common precipitin reaction. The highest dilutions in which a reaction was still noticeable corresponded to a content of 0.001 mg. per 1 cc.; consequently, the sensitivity of the tests is of the same order as precipitin reactions with proteins or bacterial carbohydrates. In the tests with the succinanilic and adipanilic immune sera the reactions were similar, but the precipitation developed more slowly and was less intense. Whilst in the case of the adipanilic sera this difference is probably due to the fact that the immune sera happened to be less active, as evidenced by the reactions with azoproteins, this explanation does not apply to the succinanilic sera which were almost as active as those for the suberanilic compound. Probably the reason for the weaker reactions is a lower precipitability of the dyes due to the shorter side chain. This assumption is borne out by the observation that other azodyes examined were far less precipitable

---

[2] *Cf.* Reference 12.

[3] Reactions were also obtained by complement fixation.

TABLE I

1/50 millimol of the dye (for instance 13.2 mg. of the resorcinoldisazo-*p*-suberanilic acid) was dissolved in 3 cc. of N/50 sodium hydroxide and 1 cc. N/50 hydrochloric acid and 1 cc. distilled water was added. The solution was centrifuged to remove traces of insoluble material. In the case of resorcinoldisazo-*p*-oxanilic acid, 1 cc. water was added in place of hydrochloric acid since it was found that the greater alkalinity was necessary to prevent flocculation upon subsequent dilution with saline.

For the precipitin tests given in Table I, 1 to 3 capillary drops of immune serum were added to 0.2 cc. of the dye solutions (prepared as described above), diluted 1:20, 1:100, and 1:500, with saline. Readings were taken after 1 and 2 hours at room temperature and after standing overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), t̲r̲. (strong trace), ±, ∓, +, +±, ++, etc.

Control tests with normal rabbit sera and some other immune sera such as precipitins for human or pig serum gave no reactions.

Immune sera for azoproteins made from	Readings taken after	Dyes made from																				
		p-Aminooxanilic acid, dilution			*p*-Aminomalonanilic acid, dilution			*p*-Aminosuccinanilic acid, dilution			*p*-Aminoglutaranilic acid, dilution			*p*-Aminoadipanilic acid, dilution			*p*-Aminopimelanilic acid, dilution			*p*-Aminosuberanilic acid, dilution		
		1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500	1:20	1:100	1:500
p-Aminosuccinanilic acid, 2 drops	1 hr.	0	0	0	0	0	0	±	+	0	0	0	0	0	0	0	0	0	0	0	0	0
	2 hrs.	0	0	0	0	0	0	+	+±	0	0	0	0	0	0	0	0	0	0	0	0	0
	Night in ice box	0	0	0	0	0	0	+±	++	±	0	0	0	0	0	0	0	0	0	0	0	0
p-Aminoadipanilic acid, 3 drops	1 hr.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	f. tr.	±	tr.	0	±	f. tr.	0
	2 hrs.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	tr.	±	t̲r̲.	0	±	t̲r̲.	0
	Night in ice box	0	0	0	0	0	0	0	0	0	0	0	0	++±	++	∓	++	±	f. tr.	+±	±	f. tr.
p-Aminosuberanilic acid, 1 drop	1 hr.	0	0	0	0	0	0	0	0	0	0	0	0	∓	∓	0	+	+	tr.	+±	++	±
	2 hrs.	0	0	0	0	0	0	0	0	0	0	0	0	∓	∓	f. tr.	+	+±	t̲r̲.	++	++	∓
	Night in ice box	0	0	0	0	0	0	0	0	0	t̲r̲.	0	0	++	++	±	++±	++±	+	++++	+++	+±

The dilutions are in terms of a solution containing 1/50 millimol of the dye in 5 cc.

than the resorcinoldisazo-suberanilic acid.[4] Azodyes made by coupling 2 mols diazotized dextro-para-aminotartranilic acid or para-arsanilic acid with 1 mol resorcinol, in dilutions of 1:100 and 1:500 of 1/50 millimol of the dye in 5 cc., gave only very weak reactions with the homologous immune sera (1, 8) upon standing for 2 hours at room temperature and overnight in the ice box. On subsequent spinning the reactions became much more evident, the precipitate appearing as large, thin flakes upon shaking up the sediment.

TABLE II

0.2 cc. of a solution of the sodium salts of the resorcinol dyes in saline (containing 1/2500 millimol in 10 cc.) were mixed with 0.05 cc. of a neutral solution of the sodium salts of the nitroanilic acids, containing 1/32 millimol in 10 cc. for the tests with the suberanilic immune serum and 1/64 millimol in 10 cc. for the tests with the succinanilic and adipanilic immune sera. 1 to 2 drops immune serum were added. The control tube contained the dye solution, immune serum and 0.05 cc. saline. The readings were taken after 1 and 3 hours at room temperature and after standing overnight in the ice box.

mmune sera for azoproteins made from	Readings taken after	Substances				
		p-Nitro-oxanilic acid	*p*-Nitrosuccinanilic acid	*p*-Nitro-adipanilic acid	*p*-Nitro-suberanilic acid	Control
p-Aminosuccinanilic acid, 1 drop	1 hr.	+	0	$\underline{+}$	$\underline{+}$	+
	3 hrs.	+	0	+	+	+
	Night in ice box	++±	0	++±	++±	++±
p-Aminoadipanilic acid, 2 drops	1 hr.	$\underline{+}$	±	0	tr.	$\underline{+}$
	3 hrs.	+	$\underline{+}$	0	$\underline{\text{tr.}}$	+
	Night in ice box	+±	+	0	±	+±
p-Aminosuberanilic acid, 1 drop	1 hr.	+±	+±	+	0	++
	3 hrs.	++	+±	+	0	++±
	Night in ice box	+++±	++±	++	0	+++±

The tests presented in the table were made with freshly prepared solutions of the sodium salts of the dyes. When the solutions (containing 1/50 millimol of the dye in 5 cc.) were kept in the ice box for several days up to a week, the precipitability with immune serum increased gradually, probably owing to a lowered dispersion of the dissolved substance. These solutions were made as described above and contained only a very small quantity of inorganic salt. For the tests they were diluted with saline in the usual way.

---

[4] In this connection the results of Hartley (13) on the precipitation of defatted proteins are worth mentioning.

From the results reported one would anticipate the possibility of eliciting anaphylactic shock in animals sensitized with azoprotein, by administration of those dyes which are distinctly precipitated by immune sera. Such was indeed the case; it is intended to describe these experiments later in detail.

Inhibition of the Precipitin Reaction.—The similarity between the precipitin reaction with dyes and those with azoproteins extends to the phenomenon of inhibition by low molecular substances with corresponding groupings. Thus neutral solutions of the sodium salts of nitroanilic and aminoanilic acids inhibited the precipitin reactions with the dyes specifically. Such results are presented in Table II. The solutions of nitro- or aminoanilic acids were not precipitated by the immune sera.

COMMENT AND SUMMARY

Experiments are described demonstrating the precipitation of azodyes by immune sera prepared by the injection of azoproteins containing the same azo component. These precipitin reactions prove conclusively the view already advanced on the basis of inhibition reactions that antibodies combine specifically with substances of small molecular weight. Although in this respect both phenomena have the same significance, the precipitin reactions with dyes are simpler in that the aid of a protein antigen is eliminated.

That specific serological precipitin reactions can take place with substances other than proteins has been amply demonstrated by studies on bacterial antigens (14) (polysaccharides (15)). The present findings show that for the precipitation with immune sera not even a high molecular weight of the reactive substance is required. Factors determining the tendency to separate out from the liquid upon combination with antibody seem to be the colloidal state of the solution and the chemical composition of the substance. With regard to the influence of chemical composition, a striking example is provided by the suberic acid dye which gives particularly strong precipitin reactions, most probably on account of its long aliphatic side chains.

The results reported may be of use for studies on the mechanism of serological precipitation.

REFERENCES

1. Landsteiner, K., *Biochem Z.*, 1920, **104**, 280.
2. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1931, **54**, 295.
3. Landsteiner, K., *K. Akad. Wetensch., Amsterdam*, 1922, **31**, 54.
4. Landsteiner, K., *J. Exp. Med.*, 1924, **39**, 631.
5. Landsteiner, K., and Levine, P., *J. Exp. Med.*, 1930, **52**, 347.
6. Klopstock, A., and Selter, E., *Z. Immunitätsforsch.*, 1928, **57**, 174.
7. Marrack, J. R., and Smith, F. C., *Nature*, 1931, **128**, 1077.
8. Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1929, **50**, 407.
9. Aschan, O., *Ber. chem. Ges.*, 1885, **18**, 2936.
10. Koller, G., *Ber. chem. Ges.*, 1903, **36**, 413.
11. Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1927, **39**, 1447.
12. Avery, O., and Goebel, W. F., *J. Exp. Med.*, 1929, **50**, 533.
13. Hartley, P., *Brit. J. Exp. Path.*, 1925, **6**, 180.
14. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, **37**, 275.
15. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, **38**, 73.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1932,
Vol. 56, No. 3, pp. 411–424]

THE INDUCED DEVELOPMENT OF NON-ACID-FAST FORMS OF BACILLUS TUBERCULOSIS AND OTHER MYCOBACTERIA*

BY FRANKLIN R. MILLER, M.D.

(From the H. K. Cushing Laboratory of Experimental Medicine in the Department of Medicine of Western Reserve University and the Medical Service of Lakeside Hospital, Cleveland)

(Received for publication, May 18, 1932)

In a previous communication (1), a description was given of an organism which was obtained from a stock culture of human tubercle bacilli, Strain H-37. This organism had the morphological characteristics of the parent strain but its growth was more rapid, and it was chromogenic, having acquired a deep yellow-orange color. On media suitable to acid-fast growth, colonies of this organism were made up of non-acid-fast rods with a scattering of acid-fast granules and rods. On more simple media, the acid-fast elements were almost entirely absent. This organism caused tuberculosis in three of five guinea pigs. It was assumed to be a non-acid-fast type of *B. tuberculosis* and was named the chromogenic H-37 strain.

The work represented in the present communication was done in order to determine whether there existed in association with the chromogenic H-37 strain an agent causing the loss of acid-fastness and, if this agent was transferable, to use it in degrading acid-fast organisms into non-acid-fast forms.

Extracts of the chromogenic H-37 organism were made and passed through a Berkefeld filter and added to the culture media on which six strains, representing four types of mycobacteria, were grown. From five of the six strains, growths which were apparently wholly non-acid-fast were obtained; the sixth strain, an organism recently isolated from human renal tuberculosis, developed non-acid-fast elements which were not readily separated from the acid-fast ones. Four of the non-acid-fast growths which appeared following treatment of the parent organism with the extract from the chromogenic H-37 organisms have by cultural methods been brought to yield acid-fast organisms again.

---

* This work was begun in the laboratories of The Rockefeller Institute for Medical Research, New York.

The history of non-acid-fast tubercle bacilli dates back almost to the original discovery of *B. tuberculosis* by Koch. In 1883, Malassez and Vignal (2) discovered non-acid-fast coccoid forms in zoogleic masses in skin lesions of a tuberculous patient and recovered acid-fast rods from them by animal passage. Ferran (3) then found that a reduction in the amount of glycerine in the media caused a reduction in the acid-fast property of the organisms and a lowering of their virulence. Auclair (4) repeated Ferran's work except that he used no glycerine in the media and was able to change a culture of tubercle bacilli to non-acid-fast coccoid and rod forms which were avirulent. He could not make this culture regain its virulence.

Further studies of tubercle bacilli during the early years (5–23), many of them dealing with the effects of variations in culture media, served to bring out the marked pleomorphism and the variability in staining reaction of this group of organisms. Of these studies it is interesting to note that Nocard and Roux were the first to find branching forms and growth by budding; that Schürmayer called attention to branching forms which were found originating in cultures of avian tubercle bacilli; d'Arrigo described extreme variation in the types of mycobacteria occurring in scrofulous lymph nodes; Marmorek stained the tiny films that first spread out from the edge of cultures of tubercle bacilli on liquid media before any of the characteristic wrinkling of the surface had taken place and found many non-acid-fast forms; and Spengler observed in sputum acid-fast bodies which he called "splitters." They were smaller than tubercle bacilli.

Koch had seen in tubercle bacilli both granules and certain refractive bodies which for a time he considered to be spores, but only a little attention had been given to either until the work of Much (24) on the granules. He found that in material such as that of cold abscesses and certain samples of sputum, in which it was impossible to demonstrate acid-fast organisms and yet which could be proved tuberculous by inoculation into animals, there were Gram-positive granules, since called Much granules. He also followed tuberculous sputum day by day and noted that when acid-fast organisms disappeared, these granules could be demonstrated. He believed that typical acid-fast bacilli could be reproduced from them. Bergel (25), on the other hand, believed that these granules should be considered as forms of degeneration. Much's work was first discussed largely from the standpoint of the relations of the granules to diagnosis, but at the present time the work is of greater interest in relation to certain general biological problems such as the nature of the life cycle of bacteria and the question of filterability. In the mycobacteria the determination of the occurrence of a non-acid-fast phase is a fundamental point in the study of whether the life cycle of these organisms is simple or complex.

The extensive literature of the past 20 years on the growth of tubercle bacilli may well be considered in connection with how much light it throws on the life cycle of these organisms. It has been analyzed recently by Löhnis (26), Sweany (27), and Kahn (28, 29). The most extensive bibliography is the recent list of 462 articles by Puntoni (30).

In 1912, Krylow (31) extended the earlier work of Marmorek and gave evidence to show that tubercle bacilli were at first non-acid-fast and Gram-negative; next developed the Gram-positive material which was always in the form of granules, and finally the acid-fastness which he thought appeared diffusely in the bacilli. The next year Wherry (32) cultivated non-acid-fast coccoid, rod, and spore-like forms from a saprophytic type of tubercle bacilli and from them regained acid-fast forms. Meirowsky (33) gave extensive illustrations of the vitally stainable granules of human and bovine tubercle bacilli and lepra organisms with studies of the growth of tubercle bacilli by budding.

More recently the studies have been more definitely along the line of a complex life cycle for the mycobacteria (34–47). In 1931, Maher summarized several years of work which had enabled him to cultivate non-acid-fast forms from old cultures of acid-fast growths. Bezançon and Philibert took the growth from the surface of liquid media, embedded it in paraffin, and stained the sections with the Ziehl-Neelsen technique and in these sections found dense networks of non-acid-fast material from which acid-fast rods and granules were growing. Ravetllat and Pla y Armengol summed up their work as indicating three phases in the life cycle of tubercle bacilli; first, a "bacterium of attack" made up of non-acid-fast forms, which were cocci, diplococci, tetrads, chains of cocci, and zoogleic masses; second, intermediate forms to be obtained from caseous material, including non-acid-fast bacilli, the Much granules, and certain intracellular forms; and third, the Koch bacillus, which they consider to be a "resisting form."

Vaudremer showed that tubercle bacilli, both human and bovine, when grown on media poor in nutritive value and without glycerine, became non-acid-fast and very pleomorphic and lost their power to produce tuberculin. In this state they ranged from a mycelium-like form to zoogleic masses. On suitable media these organisms became acid-fast again. Sweany made extensive studies of the granules and found that they could sprout and give rise to acid-fast organisms. When single-celled the granules could reproduce cultures of typical acid-fast organisms. In small doses the granules gave atypical chronic lesions and in more massive doses they gave typical tubercles. He considered them as variants in the life cycle of tubercle bacilli.

Kahn has presented the most convincing evidence of a complex life cycle of tubercle bacilli. He isolated single organisms from acid-fast cultures of tubercle bacilli, Strain H-37, planted them in microdroplets, and found that these bacilli divided into cocci which then became colonies of exceedingly small organisms which were non-acid-fast and which ultimately grew into acid-fast bacilli like the original organism. Oerskov has again raised the question of degenerative forms in the cycle as described by Kahn on the basis that similar forms can be seen in autoclaved cultures. He did not recognize that Kahn had produced tuberculosis in animals from the single cell cultures. From these studies it is clear that a complex life cycle, involving a non-acid-fast phase, has been reported repeatedly, but the difficulties involved in ruling out contaminants in a convincing manner have prevented any general acceptance of the existence of such a cycle.

Methods and Technique

The chromogenic H-37 strain was grown on plates of Petroff's egg media. At the height of the growth, or when the pigment was fully developed, the organisms were removed from the plates. Each plate yielded about 1.5 gm., which was placed in a sterile mortar. After the addition of 15 cc. of sterile saline or sterile distilled water, the material was ground for 15 minutes and then filtered through N Berkefeld candles. The filtered extracts were then placed in sterile containers and kept in the ice box. They were tested for sterility when used.

The six strains of mycobacteria used in these experiments included three human strains—Saranac H-37, T. S., and 90—, a bovine Strain B-1, a smegma Strain 74, and a Saranac strain of *B. phlei*. Three of these—H-37, T. S., and B-1—were proved to be virulent for animals. The smegma and *phlei* strains were both saprophytic, and 90 was isolated from human renal tuberculosis.

Stock cultures of all of these were grown on Petroff's egg media, and transplants were made every 4 to 6 weeks. The strain to be used for an experiment was usually of a 10 day growth. The procedure, except in two or three instances, was to add 1 to 10 cc. of filtered extract to a fresh transplant of this growth. The excess fluid was allowed to dry (in the incubator) before the culture was sealed or banded. In several experiments, a second and a third addition of the extract were made to the culture before microscopic or macroscopic changes were noted. Each time the extract was added to the culture, a control plate or tube of medium was prepared by the addition of the extract to it.

The treated cultures were transplanted in 5 days, 2 weeks, 4 weeks, and 6 weeks, with a few exceptions. The number of non-acid-fast elements which had developed prior to each transplant determined further treatment with the extract. Transplants from the originally treated cultures were made on Petroff's egg media, or on the usual laboratory media such as plain or blood agar.

After the development of many non-acid-fast forms, the organisms were grown on agar until cultures were obtained which appeared in smears to be entirely non-acid-fast. When this was achieved, they were inoculated onto media suitable for the cultivation of acid-fast growth, for example, Petroff's egg media.

Action of the Extract

When the extract was added to Petroff's egg media, the action on many cultures of the acid-fast strains mentioned was shown by typical macroscopic and microscopic changes. There was acceleration of growth of the fresh transplant within 5 days. In smears at this time many non-acid-fast organisms were seen. At 2 weeks the culture originally treated still showed more rapid growth than usual, but fewer non-acid-fast elements than at 5 days.

At about the 4th to 6th week the growth of treated cultures suddenly became fatty and moist in appearance. In some of these cultures the colonies appeared shrunken. At this time, the acid-fast elements were similar to those of old cultures: many were in the form of beaded rods, many were granules, and others only weakly acid-fast; there were also clumps of material without bacterial form. The non-acid-fast forms were often equal in numbers to the acid-fast ones and were either coccoid or bacillary. One culture, B-1 92, showed branching forms with small acid-fast granules lying along and at the ends of the organisms. From the findings it is clear that the growth of these organisms on Petroff's egg media after the use of the extract has been extremely pleomorphic. All of these strains of mycobacteria, H-37, T. S., 90, B-1, 74, and the *B. phlei* strain, have shown these peculiar changes. In the case of the strain of *B. phlei*, which normally grows rapidly on agar, the culture, treated with extract and transplanted to agar, showed acceleration of growth, loss of pigment, and many non-acid-fast forms; yet repeated transfers were necessary to eliminate the acid-fast forms.

After these peculiar macroscopic and microscopic changes had occurred, or usually after the 6th week, non-acid-fast cultures generally were grown with ease from transplants to agar.

In order to test the effect of the extract on existing acid-fast organisms, the following experiment was done. H-37 tubercle organisms were placed in 2 cc. of the extract and incubated for 96 hours. During this time, smears and cultures were made after 1 hour, 24 hours, 72 hours, and 96 hours. No increase in the non-acid-fast forms was found at these intervals, but from each transplant non-acid-fast forms were grown.

During the time that these macroscopic and microscopic changes occurred in treated cultures, the control plates remained sterile. In addition to the control plates, the sterility of the extracts was indicated by further tests on plain agar, plain broth, infusion broth, and

TABLE I

Protocol of an Experiment—(Series 72)

Culture No.	Date of transplant	Media	Type of growth	Acid-fast elements	Non-acid-fast elements	Remarks
	1931–32					
51-H		Petroff's egg	Normal	++++	0	Virulent for animals
1 (from Stock H-37, 51-H)	June 5	Long's + 10 cc. of extract (chromogenic H-37 *B. tuberculosis*)	Slight growth of pellicle in 3 wks. Plant sank to bottom. Gray-white, mucoid growth in 4 wks.	+	+++	Almost entirely non-acid-fast cocci and rods
2 (from No. 1)	July 2	Plain agar	Glistening, gray-white, pinpoint colonies in 24 hrs.	0	++++	Entirely non-acid-fast cocci and rods; rods with small, round, non-acid-fast inclusion bodies
3 (from No. 2)	July 4	Petroff's egg	Moist, fatty, yellow-white growth in 6 days	0	++++	Same as above (No. 2). Placed in ice box July 10 for 6 wks. Placed in incubator Aug. 24, still non-acid-fast. Acid-fast granules appeared by Sept. 8
4 (from No. 3)	Sept. 8	Plain agar	Glistening, gray-white, pinpoint colonies	+	+++	Biweekly transplants carried on agar through 5 mos. became and remained wholly non-acid-fast, showing cocci, rods, and diphtheroids
4*a* (from No. 3)	Sept. 8	Petroff's egg	Moist, fatty colonies, otherwise similar to mycobacterial growth	++	++	Few acid-fast rods. Many non-acid-fast rods and granules, many of these rods with acid-fast inclusion bodies

5 (from No. 4*a*)	Oct. 6	Petroff's egg	Similar to No. 4*a*, but drier	++	++	Equal distribution of acid-fast rods and granules and of non-acid-fast rods and granules
5*a* (from No. 4)	Oct. 6	Potato glycerine broth	Pellicle formation	+++	+	Mostly acid-fast rods. Few non-acid-fast rods and granules
6 (from No. 5*a*)	Nov. 20	Petroff's egg	Rapid, moist, fatty growth. Became drier, heaped up, and worm-like in appearance	++	++	Equal distribution of acid-fast rods and granules, and of non-acid-fast rods and granules
7 (from No. 6)	Jan. 14	Petroff's egg	Rapid, flat, yellow, mucoid growth in 6 days. In 2 wks., dry, waxy, heaped up appearance, typical of H-37	+++	+	At 6 days mostly non-acid-fast rods and cocci. At 2 wks. almost entirely acid-fast
Control 1	Planted June 5	Petroff's egg + 2 cc. of extract	—			No growth. Discarded Aug. 24
Control 2	Planted June 5	Petroff's egg + 2 cc. of extract	—			No growth. Discarded Aug. 24

Kendall's K medium. The K medium culture was kept sealed for 3 weeks and during that time remained sterile; it was transplanted to infusion broth and during 3 additional weeks remained sterile.

In Table I an experiment and its results are summarized, the changes being those which occurred in successive subcultures of the H-37 strain of human tubercle bacilli after the initial culture had been treated with the extract. The first culture of the experiment was planted on Long's medium and did not show the initial acceleration of growth of other treated cultures. Otherwise, the changes which occurred were similar to those in other experiments. It will be seen in the table that 4 weeks after the bacteria had been placed in contact with the extract, a non-acid-fast organism, which grew rapidly on agar, was obtained. The sequence demonstrated on the table includes the return to a morphology similar to the original strain. It also includes a notation of the continued growth on agar of the non-acid-fast forms.

From five of the six acid-fast strains employed, there were produced non-acid-fast strains. The sixth, No. 90, showed the macroscopic and microscopic changes noted above, but thus far we have been unable to eliminate all of the acid-fast forms. Four of these strains have been changed from two to six times. H-37 has yielded six non-acid-fast growths; B-1, three; T. S., two; and 74 has yielded three non-acid-fast growths.

Recently, we have also produced a non-acid-fast strain of H-37 and one of T. S., by treating cultures of these organisms with autoclaved extracts of the chromogenic H-37 strain. In all, seventeen wholly non-acid-fast growths have been obtained. All of these grew rapidly on agar and at first all but one of these growths were made up of coccoid and rod forms which were both acid-fast and non-acid-fast.

Types of Non-Acid-Fast Growth

The first isolated culture of non-acid-fast forms from a treated culture was usually a small, fine, glistening, gray-white growth. This growth, usually a transplant from Petroff's egg media to plain agar, appeared on the agar in 24 hours but was not well developed before the end of 48 hours. Microscopically, the growth usually was made up of cocci, a few of which were very large, and of rods. The cocci were often in diplococcus or tetracoccus forms, although some short chains were found. At first among the non-acid-fast forms there were found acid-fast granules and rods, as well as transition forms such as non-acid-fast rods containing acid-fast bodies, chains of acid-fast granules held together by non-acid-fast filaments, and faintly acid-fast cocci. Thus, the growth was increasingly pleomorphic.

When grown on glucose broth or infusion broth, there was a tendency to diphtheroid formation, but this did not always occur. On infusion broth and blood agar, there was also occasionally a shift back to acid-fastness even if the growth had been transplanted on agar several times.

Other types of non-acid-fast growth were observed; for instance, from a treated culture of B-1 (mentioned above), a branching non-acid-fast form was isolated. Four cultures, which were isolated as non-acid-fast cocci and rods after different periods of time on agar, changed to a more rapid, luxuriant growth which was mucoid. These growths were made up of rods containing spore-like bodies and many free spore-like bodies. When free, they were somewhat acid-fast. These bodies appeared either in the center or at the ends of the rods. One culture of chromogenic H-37, which was transferred weekly on agar for a period of 2 months, also developed a mucoid appearance. This growth showed spore-like bodies both in rods and free. Transplants of this organism were made on agar, blood agar, and Petroff's egg media. On the agar, the rods containing spore-like bodies continued to develop, the blood agar culture reverted to cocci, diphtheroids, and small rods, all non-acid-fast (the normal appearance of chromogenic H-37 on blood agar); on the Petroff's egg media, the culture returned to small non-acid-fast rods and a few acid-fast rods, and to its original appearance with deep yellow-orange pigment.

Return to Acid-Fastness

The return of the non-acid-fast organisms to an acid-fast state was a slow process. After the non-acid-fast forms had been transferred on agar for from four to eight times, they were transplanted to Petroff's egg media or other media suitable for the development of acid-fast growth. Of the seventeen non-acid-fast growths only four have returned to acid-fastness thus far. These were two cultures of H-37 (72 and 822), smegma 74, and *B. phlei*, and they have returned not only to acid-fastness but the organisms are similar to the original strain, except that they still contain a number of non-acid-fast forms. In the case of *B. phlei*, the non-acid-fast forms in the culture which returned to acid-fastness were no greater in number than those usually found. The reappearance of macroscopic morphological characteristics included the chromogenicity of *B. phlei*, and the rapid, cream-colored growth of smegma.

This return to acid-fastness has been through the development of acid-fast granules among the non-acid-fast cocci, an agglomeration of these granules into chains which resemble somewhat the beaded acid-fast rods of old cultures, the appearance of acid-fast globules within non-acid-fast rods, and finally the development of acid-fast rods. In the colonies in which acid-fast rods were developing, transition forms were always present in varying numbers. Transplants which were made when these forms predominated caused a temporary reversion to non-acid-fast forms, showing the instability of the culture.

DISCUSSION

The question of whether there exists a non-acid-fast phase of the mycobacteria is fraught with many difficulties. In this study, one source of error has been eliminated in the use of the extract from the chromogenic H-37, namely, the possibility that living organisms have been added. In the first place, all of the preparations of extract had been passed through a Berkefeld filter and the filtered extracts were all negative for viable organisms, as shown by consistently negative cultures on several standard media. Moreover, the extract was just as effective after it had been autoclaved, thus ruling out the possibility that it was a carrier of living contaminants.

Concerning the possibility of contaminations of the original cultures, or of those developing during the course of the transplantation, with some non-acid-fast saprophyte which might grow better on the simpler media but survive on media adapted to acid-fast growth, it is more difficult to bring forward convincing evidence. The facts in favor of the view that the cultures we have been working with are those of uncontaminated mycobacteria are as follows:

First, the original cultures of acid-fast organisms all showed the colony formation typical for mycobacteria and the strains all appeared to be growing in pure culture. No more non-acid-fast elements were found in smears of the original cultures than are normally found in smears of acid-fast cultures, that is to say, it required diligent search to discover even one or two non-acid-fast forms. If, however, the tubercle bacillus, at some time in its life cycle, has a non-acid-fast phase, as Kahn's observations indicate, so that not every single tubercle bacillus results from the direct fission of an acid-fast organism, then some non-acid-fast forms may be present always in any pure culture of tubercle bacilli. Second, experiments carried out in Cleveland gave the same results as in New York, so that if the non acid-fast forms were contaminants it would have to be postulated that stock cultures in the two places were carrying the same contaminants. Third, all of the organisms showed the same type of changes after the use of the extract, a change in rate of growth, in type of colony formation, and in the increased proportion of the non-acid-fast forms which were extremely pleomorphic. If contaminating saprophytes were involved which overgrew the mycobacteria on plain agar, then it would be necessary to assume that the same contamination accompanied all the strains studied in two laboratories. Fourth, frequent attempts were made by the method of dilution cultures to separate the non-acid-fast forms from the acid-fast when both were present, but we were never able, by this method, to obtain colonies which were either wholly acid-fast or wholly non-acid-fast. This is best explained by the study of the colonies themselves, for they were usually made up not only of a mixture of the two forms, but some of the non-acid-fast rods contained acid-fast particles. The development from a completely non-acid-fast culture of these transition forms, partly acid-fast and partly non-acid-fast, and the subsequent return of the culture to acid-fastness are the best evidence that the wholly non-acid-fast forms in our cultures were not contaminations.

Final evidence is to be obtained by applying the technique of the single cell culture to this problem. By this method we have already obtained a growth from a single organism isolated from an acid-fast culture. This bacillus was planted on media to which the extract had been added. The growth resulting was partly acid-fast and partly non-acid-fast. The organism grew both on agar and on Petroff's egg media and from the latter acid-fast rods have been obtained. In future experiments not only must a single cell be taken many times from the original acid-fast strain but also from the non-acid-fast subcultures until adequate data have been obtained on the limits of variation of the mycobacteria. Extensive studies also must be made on the pathogenicity of these different forms.

The evidence up to the present time, namely the similarity of morphology of the non-acid-fast growths, the regularity with which they were obtained, the transition forms in the cultures, and the fact that four of the non-acid-fast growths when transferred to appropriate media have returned to acid-fast forms showing the original type of growth, makes it reasonable to assume that the non-acid-fast organisms were derived from acid-fast ones and that the phenomenon occurs regularly.

SUMMARY

Six strains of mycobacteria,—three human strains, Saranac H-37, T. S., and No. 90, a bovine strain, B-1, a smegma strain, No. 74, and a Saranac strain of *B. phlei*,—have been made to grow as non-acid-fast organisms by the addition to the culture media of a filtered extract of the chromogenic H-37 strain of *B. tuberculosis*. The action of the extract produced acceleration of growth of the treated culture, followed by macroscopic and microscopic changes, and differentiation into non-acid-fast forms. The bacterial forms grown from these treated cultures were pleomorphic, usually consisting of cocci and small rods; but branching forms and spore-like bodies also developed.

The sterility of the extract causing the changes was demonstrated by frequent control inoculations on various media, including Kendall's K medium; and autoclaved extracts had the same effects as non-autoclaved.

After transfer to media suitable for acid-fast growths four of the strains reverted not only to acid-fastness but to their original cultural characteristics, providing evidence that the non-acid-fast forms were specific for the strain.

BIBLIOGRAPHY

1. Miller, F. R., *Science*, 1931, **74**, 343.
2. Malassez, F. R., and Vignal, W., *Arch. physiol. norm. et path.*, 1883, series 3, **2**, 369.

3. Ferran, J., *Compt. rend. Acad.*, 1897, **125**, 515; *Rev. méd.*, Paris, 1901, **21**, 1009.
4. Auclair, J., *Arch. méd. exp. et anat. path.*, 1903, **15**, 469.
5. Nocard and Roux, *Ann. Inst. Pasteur*, 1887, **1**, 19.
6. Metschnikoff, E., *Virchows Arch. path. Anat.*, 1888, **113**, 63.
7. Babes, V., and Levaditi, C., *Arch. méd. exp. et anat. path.*, 1897, **9**, 1041.
8. Cornil, A. V., and Babes, V., Les bactéries et leur rôle dans l'anatomie, Paris, Alcan, 3rd edition, 1890.
9. Schroen, *Verhandl. X internat. med. Cong., 1890*, Berlin, 1891, **2**, 120.
10. Dixon, abstracted in *Centr. Bakt.*, 1894, **15**, 492.
11. Jones, A. C., *Centr. Bakt., 1. Abt.*, 1895, **17**, 70.
12. Bruns, H., *Centr. Bakt., 1. Abt.*, 1895, **17**, 817.
13. Semmer, *Deutsch. Z. Tiermed. u. desgl. Path.*, 1895, **21**, 212; abstracted in *Centr. Bakt., 1. Abt.*, 1895, **18**, 68.
14. Craig, C. F., *J. Exp. Med.*, 1898, **3**, 363.
15. Arloing, S., *Compt. rend. Acad.*, 1898, **126**, 1398. Arloing, S., and Courmont, P., *XIII Cong. internat. méd., Sect. bactériol. et parasitol., 1900*, Paris, 1901, compt. rend., 175.
16. Schürmayer, C. B., *Verhandl. Ges. deutsch. Naturforsch. u. Ärzte*, 1898, **70**, part 2, 404.
17. d'Arrigo, G., *Centr. Bakt., 1. Abt.*, 1900, **28**, 481.
18. Marmorek, A., *Z. Tuberk. u. Heilstättenw.*, 1900–01, **1**, 444.
19. Ramond, F., and Ravaut, P., *Progrès méd.*, Paris, 1900, **12**, series 3, 429.
20. Arloing, S., and Courmont, P., *J. physiol. et path. gen.*, 1900, **2**, 82.
21. Maher, S. J., *Med. Rec.*, New York, 1904, **66**, 401; *Rep. 9th Internat. Conf. Tuberc.*, Brussels, 1910; Berlin, 1911, 350.
22. Dorset, M., *Centr. Bakt., 1. Abt.*, 1902, **31**, 305.
23. Spengler, C., *Z. Hyg. u. Infectionskrankh*, 1905, **49**, 541.
24. Much, H., *Beitr. Klin. Tuberk.*, 1907, **8**, 85, 357; 1908, **11**, 67; *Münch. med. Woch.*, 1908, **55**, 1103; *Berl. klin. Woch.*, 1908, **45**, 691.
25. Bergel, S., *Z. Tuberk.*, 1914, **22**, 343.
26. Löhnis, F., *Mem. Nat. Acad. Sc.*, 1921, **16**, 2nd memoir.
27. Sweany, H. C., *J. Am. Med. Assn.*, 1926, **87**, 1206; *Am. Rev. Tuberc.*, 1928, **17**, 53.
28. Kahn, M. C., and Torrey, J. C., *Am. Rev. Tuberc.*, 1928, **18**, 815.
29. Kahn, M. C., *Am. Rev. Tuberc.*, 1929, **20**, 150; *Ann. Inst. Pasteur*, 1930, **44**, 259.
30. Puntoni, V., *Atti III Cong. naz. microbiol.*, Milan, 1931, 175.
31. Krylow, D. O., *Z. Hyg. u. Infectionskrankh.*, 1912, **70**, 135.
32. Wherry, W. B., *J. Infect. Dis.*, 1913, **13**, 144.
33. Meirowsky, E., Studien über die Fortpflanzung von Bakterien, Spirillen und Spirochäten, Berlin, Julius Springer, 1914.
34. Meader, C. N., *Am. J. Med. Sc.*, 1915, **150**, 858.
35. Vaudremer, A., *Compt. rend. Soc. biol.*, 1921, **84**, 259; *Rev. méd.*, Paris, 1926, **43**, 419.
36. Ravetllat, J., and Pla y Armengol, R., La bacteria de la tuberculosis, Barcelona, Tipografia Catalana, 1924.

37. Bezançon, F., Philibert, A., and Hauduroy, P., *Compt. rend. Soc. biol.*, 1924, **90**, 475.
38. Bezançon, F., and Philibert, A., *Presse méd.*, 1926, **34**, 33.
39. Stockwell, W. M., *Am. Rev. Tuberc.*, 1925, **12**, 371.
40. Suyenaga, B., *Am. Rev. Tuberc.*, 1925, **12**, 260.
41. Maher, S. J., *Am. Rev. Tuberc.*, 1925, **12**, 365.
42. Maher, S. J., *Beitr. Klin. Tuberk.*, 1931, **77**, 40.
43. Karwacki, L., *Compt. rend. Soc. biol.*, 1926, **95**, 403.
44. Oerskov, J., *Compt. rend. Soc. biol.*, 1926, **95**, 1115; *Centr. Bakt., 1. Abt.*, 1931–32, **123**, 271.
45. Duffy, F. M., *Am. Rev. Tuberc.*, 1927, **16**, 330.
46. Mellon, R. R., and Jost, E., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 743; *Am. Rev. Tuberc.*, 1929, **19**, 483.
47. Mellon, R. R., *Proc. Soc. Exp. Biol. and Med.*, 1931, **29**, 206; *Tubercle*, 1931, **13**, 10.

[Reprinted from the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, August, 1932, Vol. 2, No. 1, pp. 1–10]

THE INFLUENCE OF SOME ORGAN EXTRACTS ON THE PERMEABILITY TO WATER OF SEA-URCHIN EGGS (ARBACIA PUNCTULATA)

BY GIOVANNI FAVILLI[1]

(Received for publication, June 1, 1932)

In a recent paper it was reported (1) that the fragility of certain mammalian erythrocytes is increased by testicular extract. In the last analysis such a change in erythrocytes signifies an increase in their permeability to hemoglobin. Since there was a certain amount of evidence from the work of Duran-Reynals and others (2) that the effect of testicular extract in enhancing bacterial infection may depend on increased permeability of cells and tissues, it seemed possible that testicular extract may have the general property of increasing permeability, when brought into contact with cells and tissues of various kinds.

In order to test this possibility, it appeared desirable to select a type of cell in which changes in permeability can be measured quantitatively. The egg of the sea urchin was found to be suitable material, and its permeability to water a fundamental property which has been found by Lucké and McCutcheon (3) to vary widely under experimental conditions.

Permeability to water is studied by exposing the cell to hypotonic solutions of known osmotic pressure; in such solutions water enters the cell as the result of the difference in osmotic pressure between cell contents and surrounding solution, so that the cell swells. Since the cell is spherical, a diameter is measured, and from this volume is calculated.

It has been shown by Lucké, Hartline, and McCutcheon (4) that permeability to water may be expressed as the rate of entrance of water divided by surface and pressure, or—

$$k = dV/dt/S\,(P\text{-}P_{ex})$$

---

[1] Acknowledgment is made to Dr. Balduin Lucké, of the University of Pennsylvania, for his very valuable aid in developing this investigation and in the preparation of the manuscript.

in which dV/dt is the number of cubic micra of water per minute, S is surface area in square micra, P is osmotic pressure in atmospheres of cell contents and P_{ex} is the known and constant osmotic pressure of the solution.

The details of calculation are given by the writers already referred to. In the present work, the rate dV/dt was measured by drawing tangents to the curve of swelling at both the second and third minutes; resulting values in permeability showed only slight differences between the second and third minutes.

Material and Methods

The eggs were collected from the removed ovaries of the sea urchins by washing them gently in sea water and filtering the suspension through gauze. The eggs settled promptly to the bottom of the vessel. Owing to the variation in size of the eggs from different individuals, those from a single animal were used in each experiment. As the temperature influences permeability, the level was maintained at about 15°C. during the period of manipulation. The testicle extracts were prepared from the gonads of rats and guinea-pigs according to the method described in a previous paper (1), except that sea water was used instead of 0.9 per cent saline solution. The pH of such extracts showed little variation from that of fresh sea water, i.e., pH 8.2. As control material similar extracts were made of the spleens of the same animals, for it has been shown that spleen extracts do not actively enhance infections and only slightly increase red blood cell fragility.

The eggs were placed in a Stender dish with 10 cc. of the organ extracts diluted with sea water. After a period of contact, sufficient distilled water was added to give a 40 or 50 per cent dilution of sea water. Then the size of the eggs was measured at minute intervals for six minutes by means of a microscope provided with a screw micrometer.

Because of the slight variation in size of the eggs, it was necessary to average many measurements to obtain relatively exact results. For each experiment the normal size was based on the average of forty eggs, and for each test measurement an average of six eggs was taken. The volume of the eggs was calculated from the diameter measurements. Since the eggs are very delicate material, the time of the experiment was made as short as possible; but as a further check the test eggs were measured between two sets of controls in the following order:

1. Measurement of fifteen to twenty eggs in 100 per cent sea water.
2. Measurement of six eggs in 40 to 50 per cent sea water.
3. Measurement of six eggs after contact with testicle or spleen extract in sea water diluted to 40 or 50 per cent containing testicle or spleen extract.
4. Measurement of six eggs in 40 or 50 per cent sea water.
5. Measurement of fifteen to twenty eggs in 100 per cent sea water.

The Effect of Organ Extract on Volume of Cells in Isotonic Solution

As a preliminary step it was necessary to determine whether the addition of 1 per cent testicle or spleen extract to sea water had any effect on cell volume. It was found that in every experiment, the sea-urchin eggs had increased in size after thirty minutes' exposure to such solutions. The average volume, based on 250 eggs, was in sea water $2107 \times 10^4\ \mu^3$; in sea water plus 1 per cent testicular extract, 2174×10^4; in sea water plus 1 per cent splenic extract 2190×10^4. In other words, this concentration of testicular extract increased the cells 3 per cent in volume; of splenic extract, 4 per cent. The cause of this effect is not clear.

Thus, at the time when placed in hypotonic solution, cells previously exposed to organ extracts are slightly larger than control cells. This difference introduces no difficulty in the calculation of permeability, since the initial size in both instances is known or can be extrapolated from the curve of swelling. Since permeability is calculated as rate of swelling divided by surface and pressure difference, the resulting values are valid no matter what is the initial size of the cell.

Thus it is possible to compare permeability of cells exposed to organ extracts with that of control cells and to determine whether the organ extracts have altered permeability.

This was done in twenty experiments, from which three representative experiments are selected for description of results.

Experiment No. 10

The average volume of thirty eggs was determined in ordinary (100 per cent) sea water. Cells were then exposed to testicle extract diluted 1:100 with sea water, for a period of thirty minutes. The average volume of thirty eggs in this solution was then determined. The concentration of the sea water was then reduced to 40 per cent by the addition of distilled water. Three eggs were measured at minute intervals for six minutes. The procedure was repeated and three more eggs measured. From these observations, the mean volume of six eggs was computed for each minute.

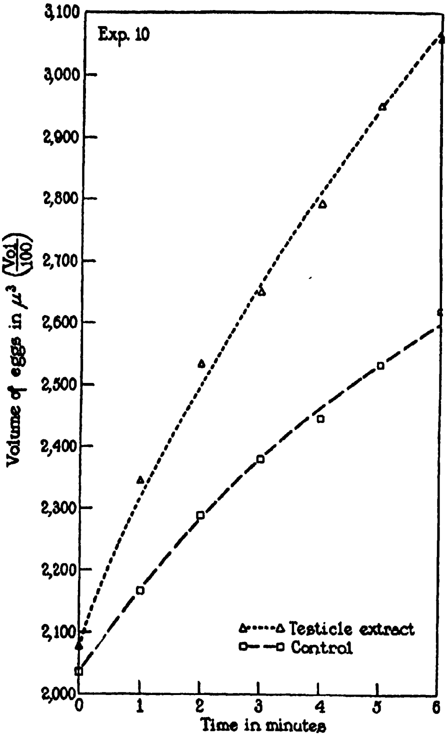


FIGURE 1

The experiment was then controlled by measuring in the same way six eggs not exposed to testicle extract.

The results are graphed in figure 1. It is seen that the rate of swelling of cells exposed to testicle extract is much greater than that of control cells. When permeability is evaluated as described above, the value obtained for cells exposed to testicle extract at the second minute is 0.088,[2] at the third minute, 0.092. The corresponding values for control cells are 0.056 and 0.050.

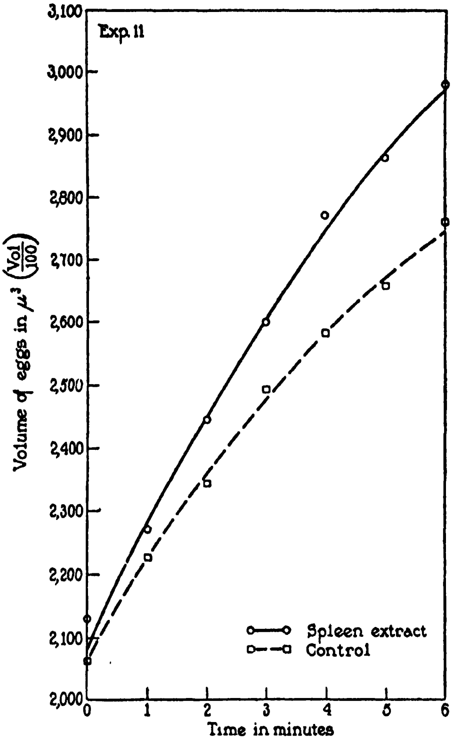


FIGURE 2

A distinct increase in permeability has thus been brought about by exposing the cells to testicle extract.

---

[2] That is, 0.088 cu.μ of water per minute enter the cell through each sq.μ of cell surface under osmotic pressure difference of one atmosphere.

Experiment No. 11

The procedure was exactly as in the experiment just described, except that spleen extract was used instead of testicle. The results are graphed in figure 2. The difference in the curves, if somewhat less striking than in the previous experiment, is nevertheless distinct.

Permeability of cells exposed to spleen extract is, at the second minute, 0.089; at the third minute, 0.088. Corresponding values for control cells are 0.066 and 0.062.

Thus it is seen that the cells exposed to extract of spleen were more permeable to water than were control cells. All of the twenty tests carried out, including more than 240 eggs treated with testicle or spleen extract, yielded comparable results. There seems to be no clear, quantitative difference between the action of the two extracts, for in some instances the swelling following contact with testicle extract was greater than that induced by spleen extract, but in others the reverse was the case.

The Effect of Time of Contact and Concentration of Organ Extracts on Sea-Urchin Eggs

Additional experiments were undertaken to determine whether more dilute testicle and spleen extracts had an influence on the permeability of the eggs.

Experiment No. 30

In this experiment, eggs were exposed to testicle extract of 1:400 dilution in sea water, and other eggs to the same concentration of spleen extract. The concentration of sea water was then reduced to 50 per cent by addition of distilled water. Nine cells in all were measured at minute intervals in each case; i.e., nine cells exposed to testicle extract, nine cells exposed to spleen extract, nine control cells.

The results are graphed in figure 3. It is seen that the two curves for cells exposed to extracts lie close together and are moderately steeper than the curve for control cells. Permeability calculations are as follows: testicle extract at second minute, 0.080; at third minute, 0.072; spleen extract at second minute, 0.076; at third minute, 0.073; controls at second minute, 0.068; at third minute, 0.065.

Thus the effect of diluting the extracts is to decrease their efficiency, but they still distinctly increase permeability.

The following additional points have been brought out in similar tests, the details of which need not be gone into: 1) With more concentrated organ extracts, 1:50 or 1:100, an appreciable swelling of the eggs is noted almost immediately after the eggs are placed in the extract, but with higher dilutions the increase in size is not evident for some minutes. 2) Testicle extract purified by precipitation with acetone retained some of its activity, but was less effective than fresh extract. 3) Blood serum induced a marked swelling in dilutions of 1:25, but in higher dilutions was less definite in its action than testicle or spleen extract. 4) Heating of spleen and testicle extracts

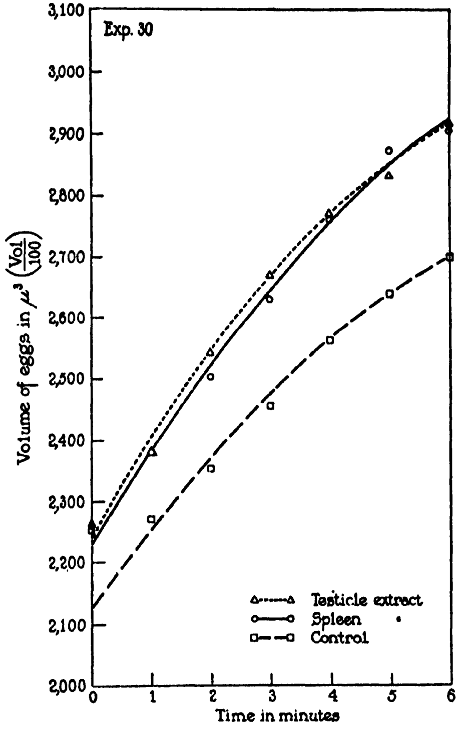


FIGURE 3

at 56°C. for thirty minutes was without effect on the extracts, but an exposure to 100°C. for three minutes seemed definitely to reduce their activity. 5) As a control to the possibility that the activity described is a common property of proteins, experiments were carried out with dilutions of 1:100 of egg albumen, gelatin, and peptone (Bacto), using the same technique as that described above. It was found that egg albumen and peptone were inactive, while gelatin tended to prevent any change, probably because of the viscosity of the fluid.

The lack of injurious activity of the organ extracts on the egg membrane was shown by the fact that eggs in which swelling had been induced regained their normal size when replaced in 100 per cent sea water and, after fertilization, showed the beginning of normal development. In fact, fertilization and cleavage occurred regularly in eggs kept in tissue extracts prepared with sea water.

DISCUSSION

In the experiments just reported it was found that with this material, sea-urchin eggs, exposure to testicle and spleen extract increases permeability to water. With erythrocytes, it had previously been found that testicle extract increases fragility, but that spleen extract does so only slightly if at all. It is not obvious, therefore, whether the mode of action of these extracts on the two types of cell is similar or not.

Nevertheless, the conclusion here drawn that organ extracts in sea water are capable of increasing the permeability of this cell to water is of considerable interest. This will become apparent when the factors known to alter cellular permeability to water are briefly enumerated. These factors have been studied chiefly in the sea-urchin egg.

This cell, at least as long as it is unfertilized and uninjured, has a rather stable permeability to water which varies only with temperature. This is true as long as the cell remains in sea water or in a solution containing adequate concentrations of bivalent cations or cations of high valence. Only when the concentration of such cations is reduced greatly below the normal is permeability increased; thus, in non-electrolyte solutions and in solutions of anions of higher valence with univalent cations, permeability is high (5). Hitherto as long as the Ca concentration was normal, no substance was certainly known to increase permeability to water (without injury). Such substances, however, appear to be present in testicle and splenic extract, since in the presence of these substances, even with normal Ca concentration, permeability of the cell to water is increased without injury.

It would be interesting to know more about the chemical nature of the active substance or substances in these extracts and how they act on the cell surface so as to decrease its resistance to the flow of water.

SUMMARY

Mammalian testicle extract and spleen extract have been studied in their effect on the permeability of cells to water. The unfertilized egg of the sea urchin, Arbacia punctulata was employed as a natural osmometer with which to measure changes in permeability.

It was found that extracts of these organs distinctly increase permeability to water in concentration of 1:100, and still distinctly but to less extent in concentration of 1:400. Blood serum has slight power to increase permeability. Several proteins were found inactive in this respect.

It has previously been reported that testicle extract increases fragility of mammalian erythrocytes. The relation of the present experiments to those on erythrocytes is discussed.

It is believed to be significant that these organ extracts increase permeability even in the presence of normal concentrations of Ca and Mg, substances which ordinarily stabilize the permeability of the cell.

LITERATURE CITED

1. Favilli, G. 1931 Effect of testicle extract on red blood cells in vitro. J. Exp. Med., vol. 54, pp. 197–206.
2. Duran-Reynals, F. 1928 Exaltation de l'activité du virus vaccinal par les extraits de certains organes. Comp. rend. Soc. Biol., T. 99, pp. 6–7.

——— 1928 The effect of extracts of certain organs from normal and immunized animals on the infecting power of vaccine virus. J. Exp. Med., vol. 50, pp. 327–340.

Duran-Reynals, F., and J. Suner Pi 1928 Exaltation de l'activité du staphylocoque par les extraits testiculaires. Comp. rend. Soc. Biol., T. 99, pp. 1908–1911.

Hoffman, D. 1931 The effect of testicular extract on filterable viruses. J. Exp. Med., vol. 53, pp. 43–50.

McClean, D. 1930 The influence of testicular extract on dermal permeability and the response to vaccine virus. J. Path. and Bact., vol. 33, pp. 1045–1070.

3. Lucké, B., and M. McCutcheon 1932 The living cell as an osmotic system and its permeability to water. Physiol. Reviews, vol. 12, pp. 68–139.

4. Lucké, B., H. K. Hartline, and M. McCutcheon 1931 Further studies on the kinetics of osmosis in living cells. J. Gen. Physiol., vol. 14, pp. 405–419.

5. McCutcheon, M., and B. Lucké 1928 The effect of certain electrolytes and non-electrolytes on permeability of living cells to water. J. Gen. Physiol., vol. 12, pp. 129–138.

Lucké, B., and M. McCutcheon 1929 The effect of valence of ions on cellular permeability to water. J. Gen. Physiol., vol. 12, pp. 571–580.

[Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, September, 1932, Vol. 17, No. 12, pages 1263–1265]

SUPRAVITAL DIFFERENTIAL COUNTING ADAPTED TO CLINICAL USE

BY ALBERT E. CASEY, M.D., AND PAUL D. ROSAHN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

The supravital method for differential counting has not been generally adopted for clinical use because of two drawbacks in the technic usually employed. The first of these is that the differential must be made shortly after preparing the blood film, since it is a common experience that after the lapse of about two hours the cells have taken up large amounts of the dye and cannot be identified. The second is the requirement of a specially constructed warm chamber which is expensive and often is not available. A method has been developed which eliminates these difficulties. This method is presented here, together with comparative results obtained by its use.

Method

The method proposed differs from that usually employed[1,2] in three respects: First, in the use of a more dilute solution of the dye; second, the preservation of the blood film in the ice box until the count can be made; and third, the substitution of a simple lighting system for the warm chamber. The stock solution of dye is made up in the usual way, 125 mg. of neutral red iodide No. 2 dissolved in 50 c.c. of absolute alcohol. The slides are coated with a dye consisting of 30 drops of the stock solution in 10 c.c. of absolute alcohol instead of the usual 100 drops.* When the blood film has been prepared and sealed with vaseline as by the ordinary method, the count may be made at once, or the preparation may be stored in the ice box at a temperature of about 4°C. until ready for the examination. In making the counts a warm chamber is unnecessary. As the source of illumination, an ordinary 40 watt frosted electric light bulb suspended on an iron stand has been found inexpensive and satisfactory.

---

* The following is the method of preparing the slides and blood films as described by Sabin: The surface of a clean slide is flooded with the diluted stock dye solution. The excess of dye is drained off the slide which is then allowed to dry. Slides stained in this manner keep indefinitely. Blood films are made by placing a small drop of blood on a clean cover-slip which is then inverted gently on the slide. The cover-slip is rimmed with vaseline and the blood films are now ready for study.

Through the use of this procedure, a preparation which has been kept in the ice box for twenty-four hours appears to be entirely similar to a fresh smear and cannot be distinguished from it; all cells have been found to be actively motile and to retain their morphologic, viable characteristics. Smears left in the open laboratory at ordinary room temperature (20°C.) were found to last about four hours or twice as long as those kept at 37° in the warm chamber. Poor results in delayed counts by this method are usually due either to improperly cleaned slides and cover-slips or to thick smears.

Comparative Results

The accuracy of delayed differential counts is evidenced by our experience summarized in Tables I and II. In a preliminary report[3] parallel observations on six male rabbits were recorded. A series of 16 smears was counted immediately after taking the blood, with the usual technic, and after twenty-four hours in the ice box, a duplicate set of smears was counted with the modifications here described. In making this comparison, 5300 cells were counted on a total of 32 smears. The means and standard errors of the means of the two series of counts are given in Table I.

That comparable results were obtained is seen from the fact that no significant difference between the respective means was obtained, and in no case was this difference equal to twice its standard error.

Further experience with this method has confirmed our original observations. Duplicate sets of smears were made on 14 normal male rabbits, 3 rabbits infected with Treponema pallidum, 3 normal men, and 3 men with active syphilis, 46 smears in all. A total of 3400 cells was counted immediately on one set of smears, following the usual technic, and 3400 cells were counted on the duplicate set after preservation in the ice box for twenty-four hours. The results are shown in Table II.

Here again, there is no significant difference between the respective means, and this difference in no case equals twice its standard error.

The method has also been employed in isolated examinations of blood from sources other than those shown in the tables. Excellent results have attended delayed differentials made on rabbits inoculated with a malignant tumor, on rabbits with severe snuffles, on men with various anemias, myelogenous leucemia, infectious mononucleosis, and syphilis.

TABLE I

Results of Immediate and Delayed Supravital Counts. First Series

	Neutrophiles		Basophiles		Eosinophiles		Lymphocytes		Monocytes	
Age of Smear in Hours	0–2	18–24	0–2	18–24	0–2	18–24	0–2	18–24	0–2	18–24
Means	59.0	57.6	3.6	4.7	0.9	0.8	23.4	24.7	12.5	12.0
Standard Error of the Mean	±2.1	±1.4	±0.4	±0.6	±0.2	±0.2	±2.2	±1.9	±1.1	±1.4

TABLE II

Results of Immediate and Delayed Supravital Counts. Second Series

	Neutrophiles		Basophiles		Eosinophiles		Lymphocytes		Monocytes	
Age of Smear in Hours	0–2	18–24	0–2	18–24	0–2	18–24	0–2	18–24	0–2	18–24
Means	53.2	54.1	4.1	4.6	2.3	2.2	30.4	29.6	9.8	9.3
Standard Error of the Mean	±1.9	±1.6	±0.6	±0.8	±0.6	±0.5	±1.9	±1.6	±0.8	±0.9

SUMMARY

A modification of the supravital technic for differential counting is here proposed which makes available to the clinician and general practitioner a method heretofore largely limited to scientific laboratories.

It renders possible accurate differential white blood cell counts with the supravital technic as long as twenty-four hours after making the preparation. This is accomplished (1) by reducing the concentration of the dye used, (2) by placing the blood smears in the refrigerator until ready for counting, and (3) by substituting a simple lighting arrangement for the warm chamber.

REFERENCES

1. Simpson, M. E.: Vital Staining of Human Blood With Especial Reference to the Separation of the Monocytes, Univ. of Calif. Pub. in Anat. **1:** 1, 1921.
2. Sabin, F. R.: Studies of Living Human Blood Cells, Bull. Johns Hopkins Hosp. **34:** 277, 1923.
3. Casey, A. E., and Rosahn, P. D.: Delayed Differential Counting of the White Blood Cells by a Modified Supravital Technique, Proc. Soc. Exper. Biol. & Med. **28:** 658, 1931.

[Reprinted from The Journal of Experimental Medicine, October 1, 1932,
Vol. 56, No. 4, pp. 483–492]

PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

VI. Action of the Associated Inhibitor on Mouse Tumors*

By JAMES B. MURPHY, M.D., and ERNEST STURM

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 14, 1932)

In previous communications evidence has been presented which indicates that an inhibitor is present in transplantable chicken tumors, together with the tumor-producing agent (1). The bases of the assumption that such an inhibitor exists are first, that the removal of a fraction from a tumor extract leaves the tumor agent in a far more active form, and second, that the inhibitor can be extracted from certain tumors in sufficient concentration to neutralize the tumor-producing property of the most active tumor extracts. It has been suggested that the two factors present in the chicken tumor, an agent which causes the malignant transformation of cells and an inhibitor which tends to balance or neutralize this agent, are related to the factors which control the growth and differentiation of normal tissues (2). The tumor agent (Chicken Tumor I) when first studied exhibited a pronounced degree of species specificity, but now shows it to a less extent. On the other hand, many active cell products are not limited in their action to the species producing them. On the basis that the inhibitor from the chicken tumor might be less limited in its effect than the agent, it has been tested on mouse tumors. The results are given in the present paper.[1]

Methods and Materials.—The following materials known to neutralize or inhibit the chicken tumor agent were tested on mouse tumors: extracts of desiccated slow-growing chicken tumor,[2] exudates from slow-growing tumors, sera from immune chickens, and sera from immunized rabbits (3). As controls to the above tests the following materials, known not to affect the chicken tumor agent, were investigated: extracts of desiccated rapidly growing chicken tumor, exudates from rapidly growing tumor, muscle, brain, and liver from normal chickens, muscle from immune chickens, and normal rabbit and chicken sera. The test solutions were prepared by thoroughly extracting 1 gm. of the tissue desiccates with 30 cc. of water, maintaining the pH at about 7 by the addition of N/10 NaOH. The extracts were then centrifuged to remove the larger particles and the supernatant fluid heated at 52°C. for 30 minutes. The latter procedure was used to destroy the tumor agent in the active extracts and for uniformity the treatment was carried out on all the controls.

---

* This investigation was carried out under the Rutherford Donation.

[1] A preliminary note on this work has been published (Murphy, Jas. B., and Sturm, E., *Science*, 1931, **74**, 180).

The transplantable mouse carcinoma utilized in the experiment was a standard tumor, known as Bashford 63. It usually gives a fairly high percentage of takes and does not often retrogress when once established. The sarcoma principally used is also a standard tumor, known as Crocker 180, characterized by the high percentage of takes it gives in practically all strains of mice, and by the fact that it is not easily influenced by procedures which increase animals' resistance to many of the other transplantable tumors. A third tumor, Mouse Sarcoma S/37, had its origin in the stroma of a transplantable adenocarcinoma, and is notable for its rapidity of growth.

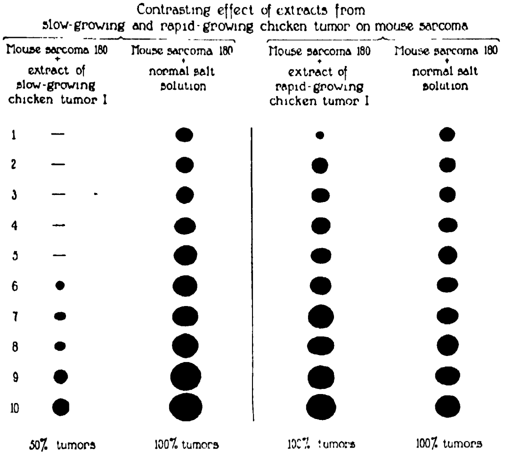
In the test with the carcinoma, grafts of the usual size were cut from the solid part of young tumors; these were placed in the extracts and nicked in several places to give a greater surface of exposure. The controls were immersed in salt solution. The contact was only for the time required to load the grafts into trocars for inoculation. With the sarcomas a suspension was made by forcing the tumors through a fine grill and adding 3 times the volume of normal salt solution. Part of this suspension was mixed with equal amounts of the test extract or fluid and 0.1 cc. inoculated into mice. For the controls the suspensions were diluted with salt solution and equal amounts inoculated. In practically all of the experiments the mice received the test inoculation in one groin and the control in the other, with additional animals inoculated with the control alone.

The Effect of Extracts of Chicken Tumor I on Transplantable Mouse Tumors

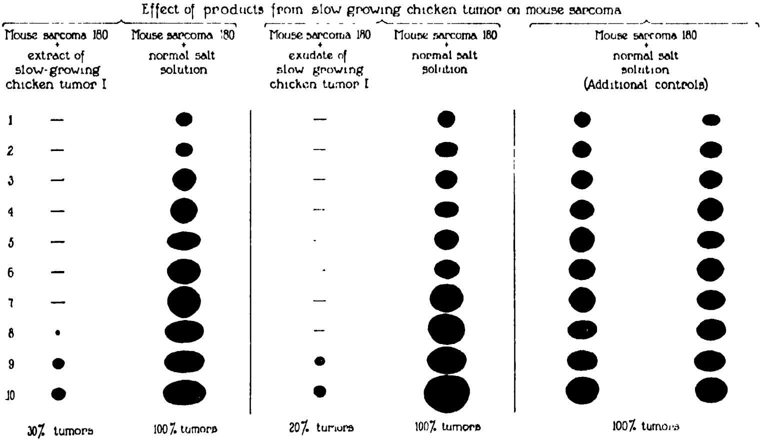
The action of chicken tumor extracts such as are known to inhibit the chicken tumor, and of others without this effect have been principally tested. So far the investigations have been confined to experiments with the three mouse tumors, for it seemed more important at

---

[2] The fact should be emphasized that not all slow-growing examples of Chicken Tumor I yield sufficient inhibitor to have the marked effect reported in this and previous papers. Extracts of desiccates of a large number of tumors were tested and those yielding the greatest concentration of inhibitor were utilized in this test. It is possible that the slow growth rate of some tumors depends on factors other than the presence of an inhibitor in the tumor.



TEXT-FIG. 1



TEXT-FIG. 2

the moment to multiply the control tests than to extend the observations to a larger variety of tumors.

Experiments.—The average individual experiment was made up of 30 mice divided into groups of 10. Two of these received inoculations of tumor plus a test fluid in one groin and in the other a control inoculation of the tumor with normal salt solution. The third group was inoculated in both groins with the tumor in salt solution. This use of double controls was done to detect a possible general effect from the local injection of inhibitors. As there was no indication of such

TABLE I

Experiment number	Material inoculated	Number of inoculations	Number negative	Negative	χ^2	*P*
				per cent		
1	Mouse Tumor 180 plus extract slow C.T.I.	131	102	77.9	126.0	0.000,000
	Mouse Tumor 180 plus salt solution	163	21	12.9		
2	Mouse Tumor 180 plus extract rapid C.T.I.	20	2	10.0	0.6	0.4
	Mouse Tumor 180 plus salt solution	60	3	5.0		
3	Mouse Tumor 180 plus exudate slow C.T.I.	18	16	88.9	61.7	0.000,000
	Mouse Tumor 180 plus salt solution	54	0	0.0		
4	Mouse Tumor 180 plus exudate rapid C.T.I.	20	0	0.0	1.0	0.3
	Mouse Tumor 180 plus salt solution	60	3	5.0		
5	Mouse Tumor 63 plus extract slow C.T.I.	46	10	21.7	0.0	1.0
	Mouse Tumor 63 plus salt solution	87	19	21.8		
6	Mouse Tumor 63 plus exudate slow C.T.I.	17	2	11.8	0.7	0.4
	Mouse Tumor 63 plus salt solution	38	8	21.1		
7	Mouse Tumor 180 plus boiled extract slow C.T.I.	39	11	28.2	0.2	0.65
	Mouse Tumor 180 plus salt solution	50	12	24.0		

action, the results of the control inoculations, whether in the test animals or in those receiving only control inoculations, are grouped together. In several experiments with materials which failed to show any inhibiting action in two tests the results of the 18 or 20 inoculations were considered sufficient.

The results of two individual experiments are given in Text-figs. 1 and 2. The first contrasts the action on Mouse Tumor 180 of a chicken tumor extract known to inhibit chicken tumors with one which had no such effect. The second shows the inhibiting action of an extract and an exudate from a slow-growing chicken tumor. The data from all of the experiments, based on over 1000 inoculations, have been brought together in Tables I and II.

TABLE II

	Number of inoculations	Number negative	Negative
			per cent
Mouse Tumor 180 plus extract normal muscle	19	1	5.3
Mouse Tumor 180 plus salt solution	32	4	12.5
Mouse Tumor 180 plus immune chicken muscle extract	9	1	11.1
Mouse Tumor 180 plus salt solution	22	1	4.5
Mouse Tumor 180 plus normal chicken serum	29	3	10.3
Mouse Tumor 180 plus salt solution	60	4	6.7
Mouse Tumor 180 plus immune chicken serum	19	0	0.0
Mouse Tumor 180 plus salt solution	40	1	2.5
Mouse Tumor 180 plus normal rabbit serum	20	0	0.0
Mouse Tumor 180 plus salt solution	40	1	2.5
Mouse Tumor 180 plus immune rabbit serum	20	0	0.0
Mouse Tumor 180 plus salt solution	40	1	2.5

In addition to the figures for the tests and controls of each group, we have included an analysis of the principal experiments (Table I) by applying the χ^2 test with its corresponding probabilities (4). This method tests the independence of the proportionate differences between the two groups under comparison. P is a measure on the scale of 0 to

1 of the probability that the deviations from the theoretical frequencies may be reasonably supposed to be due to the errors of sampling. If P is below 0.02 we may consider that a real effect had been produced. It will be noted that Experiments 1 and 3 in Table I show an unquestionable difference between the test and the control inoculations, which may be considered proof of an inhibiting action of the extracts and exudates of slow-growing tumors. In Experiments 2 and 4, where the extracts and exudates were obtained from rapidly growing chicken tumors, no inhibiting action is indicated. In Experiment 5 the unusual value of $P = 1$ was obtained, but undoubtedly the extract in this case had no effect on the Tumor 63. The destruction of the inhibitor by boiling is shown by the results in Group 7.

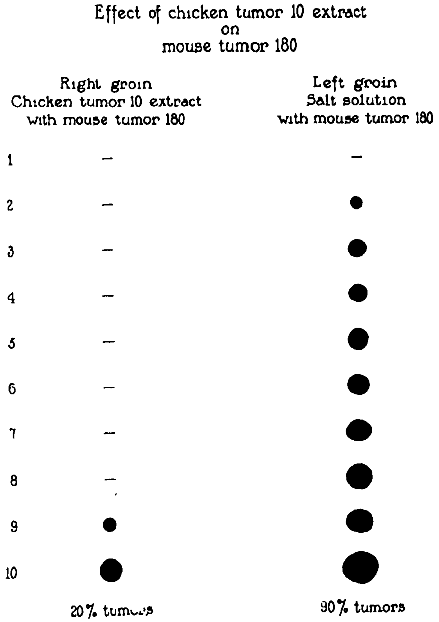
Table II lists a number of experiments based on smaller numbers, in which tests with extracts from muscle and with normal and immune sera gave negative results.

In addition to the experiments included in the tables a few tests were made with extracts of desiccated brain and liver of normal chickens, which were found to be without effect on Mouse Tumor 180. Extensive tests with Mouse Tumor S/37 failed to show any influence on its growth after treatment with extracts known to inhibit chicken tumors and Mouse Tumor 180.

The number of tests is sufficiently large to leave little doubt that the extracts of certain relatively slow-growing chicken tumors and the exudates from such tumors have a definite inhibiting action on a transplantable mouse sarcoma and are without effect on a mouse carcinoma. The number and variety of the controls very largely eliminate the possibility that the result is due to injury from some incidental enzyme or chemical. Perhaps the best indication of this is the failure of products of the rapidly growing tumors to exert any effect.

Effect of Products of Chicken Tumor X on Mouse Tumor

Andrewes (5) has reported that the serum from chickens bearing either of two slow-growing fibrosarcomas for at least 5 months will neutralize the tumor agents of Chicken Tumor I and the tumor known as Mill Hill 2. He considers this property in the nature of a virus antibody, which would indicate a common or closely related etiologic agent for these tumors. The preceding experiments show that the antibodies developed against the tumor agent either in chickens showing a certain amount of natural resistance or in rabbits actively immunized, while capable of neutralizing the chicken tumor agent, are without effect on the mouse tumors. The possibility that the neutralizing property of the sera described by Andrewes represents the action of an inhibiting factor instead of an antibody has not been eliminated. The following experiments with Chicken Tumor X probably throw some light on the question, as the sera from fowls bearing this tumor were used by Andrewes in his experiments referred to above.



TEXT-FIG. 3

Experiment.—Chicken Tumor X has been used as the source of extracts. This tumor, a transplantable fibrosarcoma, derived from a spontaneous tumor, has been under investigation in this laboratory for the last 5 years. As a rule it grows very slowly, often requiring from 8 months to over a year to kill the animal. During this period it attains enormous size. At times it has grown more rapidly but even at these periods metastases have taken place with great rarity. It is transmitted with difficulty by desiccates and only one doubtful result has been obtained with filtrates.

The methods used were the same as those for the preceding group of experiments. The desiccates were prepared from tumors of about a year's growth and the extracts tested on Mouse Tumor 180. The results are presented in Table III and an individual experiment in Text-fig. 3.

It is evident from the figures in Table III, based on five experiments in which 138 inoculations were made, that Chicken Tumor X yields an inhibitor for Mouse Tumor 180. The percentages of complete inhibition are not as striking as those with the inhibitor from Chicken Tumor I. It might have been expected that the inhibitor from the former tumor would be less potent, as it is associated with a tumor agent of relatively low grade activity.

TABLE III

	Number of experiments	Number of inoculations	Number negative	Negative
				per cent
Mouse Tumor 180 plus extract of Chicken Tumor X..........	5	69	41	59.5
Mouse Tumor 180 plus salt solution . . .	5	69	4	5 8

DISCUSSION

The principal question suggested by the findings is whether the inhibiting action exerted by the extracts of certain desiccated chicken tumors represents a definite, specific force or whether it represents an incidental result, devoid of importance. It is difficult to reconcile the lack of an inhibiting element in extracts from rapidly growing tumors with the latter view. While as yet sufficient evidence is not available for a final conclusion, there are certain facts which justify a tentative interpretation. Perhaps the most important of these is that the inhibitor from a chicken sarcoma acts on a mouse sarcoma and not on a mouse carcinoma; but this observation must be extended to a large variety of tumors before we can accept the reaction as specific. The absence of demonstrable effect of the inhibitor on another mouse sarcoma (S/37) may be due to the unusual malignancy of this tumor, or there may be some question as to the nature of this growth which is supposed to be a sarcoma but had its origin in the stroma of a carcinoma.

If the inhibitor is a definite factor its possible relationship to antibodies must be considered. The fact that antibodies developed against the chicken tumor have no effect on the mouse sarcoma while inhibitor derived from the tumor does retard these growths, suggests a difference. Andrewes (5) has expressed some doubt that a substance identical with the serum antibody is responsible for the inhibition of the growth of mammalian tumors on the ground that the antibodies seem to act against the filtrate, not against the cells. This point in our opinion requires closer scrutiny, for the inhibitor has but a doubtful effect on the chicken tumor cells and yet does act on mouse tumor cells. We know from the present findings that one of the tumors used in Andrewes' experiments yields an inhibitor for mouse tumors as well as for chicken tumors, and it seems not inconceivable that the sera of fowls bearing the tumor would also contain the inhibiting factor.

It may be suggested tentatively that the property of extracts from certain relatively slow-growing strains of Chicken Tumor I, and Chicken Tumor X, by virtue of which the chicken tumor agents are neutralized and the growth of mouse sarcoma cells is inhibited, represents a definite factor distinct from the usual type of antibody.

SUMMARY

Water extracts of desiccates of certain relatively slow-growing strains of Chicken Tumors I and X, or the exudates from such tumors, definitely inhibited the growth of a mouse sarcoma (Crocker 180), and were without effect on a mouse carcinoma (Bashford 63) or Mouse Tumor S/37, a rapidly growing sarcoma derived from the stroma of a carcinoma. Extensive control tests with extracts from rapidly growing chicken tumors, and from tissues of normal and immune chickens showed no inhibiting action. There was no demonstrable action on the mouse tumors of sera from immunized rabbits, which neutralize the chicken tumor agent, nor of the sera from chickens highly immune to the chicken tumors.

REFERENCES

1. Murphy, Jas. B., Helmer, O. M., Claude, A., and Sturm, E., *Science*, 1931, **73**, 266. Murphy, Jas. B., *Tr. Assn. Am. Physn.*, 1931, **46**, 182. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56**, 107. Sittenfield, M. J., Johnson, A. S., and Jobling, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 517.

2. Murphy, Jas. B., Sturm, E., Claude, A., and Helmer, O. M., *J. Exp. Med.*, 1932, **56**, 91.
3. Murphy, Jas. B., Sturm, E., Favilli, G., Hoffman, D. C., and Claude, A., *J. Exp. Med.*, 1932, **56**, 117.
4. Yule, G. U., *J. Roy. Statistical Soc.*, 1922, **85**, 95.
5. Andrewes, C. H., *J. Path. and Bact.*, 1931, **34**, 91; 1932, **35**, 243.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, November 1, 1932,
Vol. 56, No. 5, pp. 705–709]

PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

VII. SEPARATION OF THE ASSOCIATED INHIBITOR FROM TUMOR EXTRACTS*

BY JAMES B. MURPHY, M.D., AND ERNEST STURM

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 30, 1932)

The presence of an inhibitor or balancing factor associated with the causative agent in a chicken tumor was first indicated by the fact that the tumor-producing activity of the agent was greatly increased when a fraction of the tumor extract or filtrate was removed. This indication was confirmed by the demonstration that certain relatively slow-growing chicken tumors yielded the inhibitor in sufficient concentration to neutralize the tumor agent in the most active extracts (1). It has seemed desirable in the further study of the inhibitor fraction to secure it in greater concentration and freed so far as possible from the many other products contained in the crude tumor extract. The present paper is a report of the first step in this direction.

The Release of Chicken Tumor I Inhibitor after Adsorption on Aluminum Hydroxide

Previous experiments have shown that the most effective way of freeing a tumor extract of the inhibitor was to treat it with aluminum hydroxide (Willstätter Type C) (2), which under proper conditions appears to adsorb the inhibitor factor almost entirely but very little of the tumor-producing agent. The possibility of releasing the inhibitor from the aluminum hydroxide, thus freeing it from some of the impurities, was tested in the following experiments.

Experiment.—The material utilized in the experiments consisted of extracts of desiccated Chicken Tumor 1 known to yield sufficient inhibitor to neutralize active tumor filtrates, and to inhibit the growth of a mouse sarcoma (Crocker 180)

---

* This investigation was carried out by means of the Rutherford Donation.

(4); and extracts of tumor desiccates known to be without effect on the chicken tumor or the mouse sarcoma. These materials were prepared by thoroughly extracting 1 gm. of the dry tumor powder with 30 cc. of water. After centrifuging out the larger particles the supernatant fluid was filtered through filter paper, adjusted to about pH 7.2 and 20 cc. of this was shaken with an equal volume of Willstätter Type C aluminum hydroxide. The aluminum was then separated out

TABLE I

Release of Chicken Tumor Inhibitor after Adsorption on Aluminum Hydroxide

Materials inoculated	No. of inoculations	No. negative	Average size of tumors	Negative
			cm.	*per cent*
Material from slow-growing tumors				
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated extract slow-growing tumor	19	18	0.6 x 0.4	94.4
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated aluminum supernatant fluid of slow-growing tumor extract	19	1	2.3 x 1.8	5.3
0.2 cc. active C. T. I filtrate plus 0.2 cc. phosphate release from aluminum	19	18	1.1 x 1.0	94.4
0.2 cc. active C. T. I filtrate plus 0.2 cc. water	36	0	2.3 x 1.9	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. M/15 basic sodium phosphate	14	0	2.2 x 1.9	0
Material from rapidly growing tumors				
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated extract rapidly growing tumor	9	0	1.7 x 1.4	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated aluminum supernatant fluid of rapidly growing tumor extract	9	0	2.1 x 1.6	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. phosphate release from aluminum	9	0	1.9 x 1.6	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. water	18	0	2.4 x 1.8	0

by centrifugation and washed with 10 cc. of distilled water 2 to 3 times. The wash waters were then discarded and the aluminum deposit was shaken up with 10 cc. of M/15 basic sodium phosphate at pH 9, the released material having a final pH of 8. The aluminum was removed and discarded. The eluate was heated at 52° for 30 minutes to inactivate any tumor agent which might have come through.

The tests consisted of the injection of mixtures in equal amounts of the eluate and active tumor filtrate, controlled by the injection of the active filtrate diluted with the heated aluminum supernatant fluid, with water and with M/15 basic sodium phosphate buffer. The inoculations were made with 0.4 cc. of the mixtures intradermally, so that each chicken received the test material and those of the controls as well. In the first group of experiments the extracts were prepared from tumor desiccates known to yield the inhibitor factor and in the second group from desiccates of rapidly growing tumors, known to yield no inhibiting substance. The results of 11 experiments with 152 inoculations are given in Table I.

It is evident from Table I that the inhibiting factor may be adsorbed on aluminum hydroxide and released with basic sodium phosphate. While the experiments were not designed to test quantitative differences between the original extract and the released material, yet they indicate that there is certainly no very great loss in the inhibiting property after adsorption and release. The failure of the heated extracts from the rapidly growing tumor to influence materially the tumor production by active filtrates is in line with previous observations and, as might be expected, there is no evidence of inhibiting action by the released material. That the phosphate in the released fluid is not responsible for the inhibiting action is shown not only by the controls, in which the phosphate was added to the active filtrate, but also by the tests with the released material from the rapidly growing tumor extract.

Release of Inhibitor from Chicken Tumor X after Adsorption on Aluminum Hydroxide

Chicken Tumor X, a very slow-growing fibrosarcoma, has proved a source of an inhibitor for Chicken Tumor I and Mouse Tumor 180 (3, 4). The inhibitor, judged by previous tests, is less potent than that from Chicken Tumor I, which might be due to the fact that the agent with which it is associated is less vigorous.

Experiment.—The technical procedure used in these experiments was the same as that described above. The source of the fluids tested for inhibiting action was extracts of desiccates prepared from Chicken Tumor X, which had required 9 months to a year to reach a large size. Beside the heated full extract, the supernatant fluid after the extract had been adsorbed out with aluminum hydroxide and the material released from the aluminum by treatment with basic sodium phosphate were tested against an active extract of Chicken Tumor I. In each instance the fluids to be tested were heated at 52° for 30 minutes. The controls included the active extract diluted with salt solution and with basic sodium phosphate. The results of the intradermal inoculations, in which each chicken received the control injections and the test inoculations as well, are given in Table II.

TABLE II

Release of the Inhibitor from Chicken Tumor X after Adsorption of Aluminum Hydroxide

(Based on 8 experiments)

Materials inoculated	No. of inoculations	No. negative	Average size of tumors	Negative
			cm.	*per cent*
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated extract C. T. X	21	18	0.9 x 0.8	85.7
0.2 cc. active C. T. I filtrate plus 0.2 cc. heated aluminum supernatant fluid of C. T. X	13	0	1.9 x 1.6	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. phosphate release from aluminum	16	14	0.4 x 0.4	87.5
0.2 cc. active C. T. I filtrate plus 0.2 cc. M/15 basic sodium phosphate	10	0	1.8 x 1.5	0
0.2 cc. active C. T. I filtrate plus 0.2 cc. salt solution	22	0	2.2 x 1.5	0

The results show conclusively that Chicken Tumor X contains an inhibiting factor capable of neutralizing the agent of Chicken Tumor I, and that the inhibitor is adsorbed by aluminum hydroxide and can be released from this combination. There is no indication of loss in potency of the inhibitor by this manipulation, a finding similar to that noted in the first group of experiments.

DISCUSSION

There is no doubt that it is possible to adsorb out practically all of the tumor agent from a filtrate with aluminum hydroxide when a large amount of the colloid is used (5). With the proportions used in these and the earlier experiments reported comparatively little of the agent is taken up, but the inhibiting factor seems to be entirely removed and to be recoverable from the aluminum. The amount of inhibitor lost by this treatment has not yet been accurately determined, but certainly effective quantities are present in the eluate.

SUMMARY

The inhibiting factor present in certain relatively slow-growing strains of Chicken Tumor I and in Chicken Tumor X is adsorbed from extracts of the desiccated tumors by aluminum hydroxide (Willstätter Type C) and can be released in effective quantities from this combination by treatment with basic sodium phosphate.

BIBLIOGRAPHY

1. Murphy, Jas. B., Helmer, O. M., Claude, A., and Sturm, E., *Science*, 1931, **73**, 266. Murphy, Jas. B., *Tr. Assn. Am. Physn.*, 1931, **46**, 182. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56**, 117; *Science*, 1931, **74**, 180; 1932, **75**, 540; *J. Exp. Med.*, 1932, **56**, 107. Sittenfield, M. J., Johnson, A. S., and Jobling, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 517.
2. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, 1923, **56**, 149, 1117.
3. Murphy, Jas. B., and Sturm, E., *Science*, 1931, **74**, 180.
4. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56**, 483.
5. Murphy, Jas. B., Sturm, E., Claude, A., and Helmer, O. M., *J. Exp. Med.*, 1932, **56**, 91.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, November 1, 1932,
Vol. 56, No. 5, pp. 711-717]

PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

VIII. THE EFFECT OF TESTICLE EXTRACT ON THE RATE OF GROWTH OF CHICKEN TUMOR I*

BY ERNEST STURM AND F. DURAN-REYNALS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 30, 1932)

The observation that testicle extract markedly enhances the infectivity of certain viruses and bacteria, reported by one of us (Duran-Reynals (1)) has been confirmed and extended by a number of investigators (2). It has been further shown that testicle extract exerts an inhibitory influence on the growth of a transplantable epithelioma of the rabbit (3) and to a less extent on a sarcoma of the mouse (4). It is of interest to know what effect testicle extracts will have on the chicken neoplasms, transmissible by cell-free extracts.

Recently Hoffman, Parker and Walker (5) have published the results of a similar investigation in which they report evidence of the enhancement of the chicken tumor agent by rabbit testicle extract, but none by that from rooster testicle. This evidence is based on two positive experiments, but the same testicle extract was used in both tests. They draw no conclusions as to the nature of the tumor agent from these results, but give the impression that further investigation with the method might lead to some conclusions on the question. During the last 3 or 4 years a considerable amount of data had been accumulated in this laboratory, but had not been published at the time the article referred to appeared. Since our results differed from those reported by the above authors, we deferred publication until additional tests could be made with certain minor modifications in technique suggested by their paper.

---

* This investigation was carried out by means of the Rutherford Donation.

Materials and Methods

The testicle extracts were prepared by removing the glands of rat, rabbit or rooster, trimming them of all adhering tissue and removing the capsule. The tissue was then ground with sand in a mortar along with an equal volume of physiological salt or Ringer's solution. After centrifuging down the heavier particles, the supernatant fluid was decanted, filtered through a Berkefeld V candle and used in the tests.

The testicle extracts of rat, rabbit and rooster and the purified bull testicle extract were tested with (1) the active extract of desiccated Chicken Tumor I, (2) the concentrated Berkefeld filtrate of fresh Chicken Tumor I and (3) suspensions of the tumor cells.

The Effect of Testicle Extract on the Activity of Extracts of Chicken Tumor I Desiccates

These experiments, both in the method of preparation of the testicle extracts and the location of inoculations, followed closely the technique used in testing the action of the enhancement factor on viruses and bacteria which has been reported by one of us (Duran-Reynals).

Experiments.—The tumor extract was prepared by thoroughly extracting 1 gm. of finely powdered desiccate of Chicken Tumor I with 50 cc. of distilled water and adding enough N/10 NaOH to render the solution either neutral or slightly alkaline. A pH of 7.2 to 7.4 was usually maintained in all extractions. The emulsion suspension was thoroughly mixed by passing it in and out of a large hypodermic syringe fitted with a 12 gauge lumbar puncture needle for several minutes. After centrifugation the supernatant fluid was filtered through moistened, coarse filter paper and divided into 5 portions. To each of the first 4 portions was added an equivalent amount of testicle extract to be tested, while to the remaining 5th portion an equal volume of either salt or Ringer's solution was added—the latter to serve for the activity control injections. In some of the tests the testicle extracts were diluted 10 times with Ringer's solution, but this modification of the method had no effect on the results. In consequence all of the experiments are grouped together. The solutions of the testicle and chicken tumor extracts were thoroughly mixed and allowed to stand at room temperature for the length of time necessary to complete the injection. This period usually varied from 20 to 40 minutes. 0.4 cc. of each of the respective mixtures, including the control, was injected intradermally into a normal chicken. By this means each chicken received the 4 test injections and the control for activity. It was noted that the bleb resulting from the intradermal injection of the solutions containing testicle extract flattened in a very few minutes after injection, while those without testicle extract required considerably longer to spread into the surrounding tissues.

Weekly measurements of the resulting tumors were made. The figures on the size of the tumors recorded for each chicken were the last before the tumors broke through into the subcutaneous tissue or coalesced in the skin. This method in our opinion gives a fair picture of the results. As each bird carried all of the test inoculations as well as the control injections, the variation in susceptibility of individual fowls and in the activity of the different tumor extracts is minimized. The chickens used in the tests were of the Plymouth Rock type, adults of uncertain age, bought in the open market. Fresh extracts were used in each group. The outcome of 4 experiments, based on 72 inoculations, is given in Table I.

There is no evidence of enhancement of the chicken tumor agent by the various testicle extracts with the methods used in these experiments. Nor do we find any inhibiting action, with the possible exception of the tests with purified bull testicle; but the numbers here are too small to be significant. Furthermore, at no time during the course of the experiments was there any material evidence of enhancement or inhibition in the development of the tumors.

TABLE I

The Action of Testicle Extract on Chicken Tumor I Extracts

(4 experiments)

Chicken Tumor I extract	No. of inoculations	No. of tumors	Average size of tumors
			cm.
Rat testicle extract	20	20	2.5 x 2.0
Rabbit testicle extract	8	8	2.3 x 1.8
Rooster testicle extract	16	16	2.5 x 2.0
Purified bull testicle extract	4	4	1.6 x 1.1
Salt solution (control)	24	24	2.3 x 1.9

The Effect of Testicle Extract on the Tumor Production by a Concentrated Berkefeld Filtrate of Chicken Tumor I

As a further test of the possible effect of testicle extract on the chicken tumor agent, filtrates of fresh tumor extracts were utilized in the next group of experiments instead of the extracts of tumor desiccate, which were the source of the agent in the preceding group.

Experiments.—Berkefeld filtrates were prepared by grinding 25 gm. of mashed chicken tumor tissue in a mortar along with a quantity of sterile sand, diluting with 500 cc. of distilled water and adding N/10 NaOH to bring the pH of the solution to 7.2–7.4. The solution was shaken for 20 minutes in a stoppered flask, centrifuged and the supernatant fluid passed through Berkefeld V candles under 25 lbs. of air pressure. 300 cc. of the filtrate was collected and concentrated to 40 cc. in alundum thimbles lined with 8 per cent collodion membranes. The testicle extracts were prepared in the same manner as in the previous experiments.

For the test, 0.4 cc. of mixtures of equal parts of the concentrated filtrate and testicle extracts was injected intradermally in chickens. For the control of activity an equal amount of the concentrate diluted with Ringer's solution was injected into each chicken. Fresh testicle extracts and tumor filtrates were prepared for every experiment. The results of two groups of tests are given in Table II.

While the number of fowls inoculated in this group is not large, yet they show a uniform lack of any effect of the testicle extracts on the size of tumors produced by tumor filtrates.

TABLE II

The Action of Testicle Extract on Fresh Concentrated Berkefeld Filtrate of Chicken Tumor I

(2 experiments)

Concentrated Berkefeld filtrate of Chicken Tumor I	No. of inoculations	No. of tumors	Average size of tumors
			cm.
Rat testicle extract	6	6	1.8 x 1.3
Rooster testicle extract	6	6	1.8 x 1.6
Salt solution (control)	6	6	2.1 x 1.5

The Effect of Testicle Extract on the Size of Tumors Resulting from the Inoculation of Tumor Cells

As stated above, testicle extract definitely inhibits the growth of a transplantable rabbit tumor and to a less extent of a certain transplantable mouse tumor. In these instances the tumors are transferred only by cells. To parallel these experiments more closely we have tested the effect of testicle extract on the size of tumors produced by the chicken tumor cells.

Experiments.—The chicken tumor cell suspensions were made by taking 1 cc. of freshly mashed chicken tumor tissue, passing it through a fine wire gauze mesh under slight pressure and suspending the cells in 5 cc. of Ringer's solution. For the injection a mixture was made of equal parts of the cell suspension and the respective testicle extracts. The total volume injected into each area of a normal chicken was 0.4 cc. The results of these experiments are shown in Table III.

From the figures given in Table III there seems to be no enhancement of the growth rate of tumors resulting from mixtures of chicken tumor cells with testicle extract from four different species.

The Effect of Testicle Extract on the Development of Tumors in Young Fowls

Hoffman, Parker and Walker (5), in their experiments referred to above, used 8 weeks old chicks and reported enhancement with extracts of rabbit testicle (2 experiments with the same extract), but none with that from the rooster. In order to check the possibility that the age of the fowl affects the results our experiments have been repeated using chicks 6 to 8 weeks old.

TABLE III

The Action of Testicle Extract on Chicken Tumor I Cells

(3 experiments)

Chicken Tumor I cell suspension	No. of inoculations	No. of tumors	Average size of tumors
			cm.
Rat testicle extract	6	6	2.0 x 1.4
Rabbit testicle extract	14	14	2.2 x 1.6
Rooster testicle extract	3	3	2.0 x 1.4
Purified bull testicle extract	2	2	1.9 x 1.5
Salt solution (control)	12	12	2.3 x 1.5

Experiments.—The tests were confined to the effect of rat and rabbit testicle extracts, but these were tested on both tumor cells and extracts of desiccated tumor, the inoculations being made intradermally. The conditions of the experiments were the same as in the preceding groups, except that young chicks were used. The results of these experiments, with fresh extracts for each experiment, are given in Table IV.

A further experiment was carried out with 8 weeks old chicks, in which a mixture of chicken tumor cells and rat testicle extract was injected intramuscularly in one side, and in the other the tumor cell suspension appropriately diluted for control. The measurements of the resulting tumors suggested some enhancement in 3 of the 4 fowls. They were killed at the height of the experiment and the true measurements made at autopsy showed no material difference between the two sides. This was verified by the weight of the two tumors in the one chick showing the greatest difference in the crude measurements.

From these last tests it seems that there is no evidence of enhancement of tumors by testicle extract in young chicks, thus confirming the results we had obtained in adult birds.

TABLE IV

The Action of Testicle Extract on Chicken Tumor I
8 Weeks Old Chicks Injected

(3 experiments)

Material injected	No. of inoculations	No. of tumors	Average size of tumors
			cm.
Chicken tumor cells with:			
Rat testicle extract	4	4	3.3 x 2.5
Rabbit testicle extract	4	4	3.2 x 1.9
Salt solution (control)	8	8	3.2 x 2.3
Chicken tumor extract with:			
Rat testicle extract	7	7	2.3 x 2.2
Rabbit testicle extract	7	7	2.1 x 1.6
Salt solution (control)	14	14	2.2 x 1.7

DISCUSSION

With testicle extract we have a factor or factors which augment the infection of viruses and bacteria on the one hand, and on the other seem to retard the growth of certain transplantable tumors. With this evidence it might be supposed that the action of testicle extract on the chicken tumor would give suggestive information as to the nature of the causative agent. However, since the testicle factor enhances infective agents (6) by increasing tissue and cell permeability, and since it enhances the action of certain toxins (7), it would appear that no deduction could have been drawn as to the nature of the chicken tumor agents even if the experiments had shown a definite enhancement. As a matter of fact we have found no evidence of definite enhancement or inhibition with the various testicle extracts investigated. There may be some simple explanation in this failure such as the structure of the fowl's skin, but this does not seem likely since the ability of the testicle extract to bring about an increase in the area of spread of injected materials is just as evident in the chicken skin as in that of the mammal. We see no immediate explanation of the difference in the results of our experiments and those of Hoffman, Parker and Walker.

SUMMARY

Extracts prepared from the testicle tissue of the rat, rabbit, fowl or bull, injected together with extracts of Chicken Tumor I or with cells of this tumor, showed no definite effect of either enhancement or inhibition as concerned the resulting tumors.

BIBLIOGRAPHY

1. Duran-Reynals, F., *Compt. rend. Soc. biol.*, 1928, **99,** 6; *J. Exp. Med.*, 1929, **50,** 327. Duran-Reynals, F., and Suñer-Pi, J., *Compt. rend. Soc. biol.*, 1928, **99,** 1908.
2. McClean, D., *J. Path. and Bact.*, 1930, **33,** 1045. Hoffman, D. C., *J. Exp. Med.*, 1931, **53,** 43. Pijoan, M., *J. Exp. Med.*, 1931, **53,** 37.
3. Duran-Reynals, F., *Science*, 1930, **72,** 1876; *J. Exp. Med.*, 1931, **54,** 493.
4. Tanzer, R. C., *J. Exp. Med.*, 1932, **55,** 455.
5. Hoffman, D. C., Parker F., Jr., and Walker, T. T., *Am. J. Path.*, 1931, **7,** 523.
6. Hoffman, D. C., and Duran-Reynals, F., *J. Exp. Med.*, 1931, **53,** 387.
7. McClean, D., *J. Path. and Bact.*, 1930, **33,** 1045.

[Reprinted from THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, October 8, 1932, Vol. 99, No. 15, pp. 1244–1246]

IMMUNITY TO HUMAN AND PASSAGE POLIOMYELITIS VIRUS*

BY SIMON FLEXNER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

A considerable body of knowledge relating to active immunity to the virus of poliomyelitis has grown up since the condition was first described in 1910 (1). This paper will deal briefly (2) with several aspects of this immunity problem.

In the first place, attention will be given to the immunity produced in monkeys by the subcutaneous or the intradermal injection of specimens of virus passed through many monkeys or derived immediately from fatal cases of human poliomyelitis. A comparison of the quality of the immunity in the two cases will be made according to the resistance against infection and the demonstration of antiviral or neutralizing properties in the blood sera.

A consideration of this problem leads directly to questions of differences or variants of the virus as it exists in epidemics arising in separate years or in places widely removed from one another, and to changes produced in the human virus by monkey passages.

In the next, attention will be given to active immunity as it arises especially in monkeys subjected to inoculation. In this connection reference may be made to the fact, often observed but little commented on, that non-infective doses of the virus, when they are introduced into the central nervous system, tend not to lead to immunity. While one or more clinically ineffective doses of virus injected into the skin or subcutis increase the resistance or actively immunize monkeys, such non-infective injections made intracerebrally or intracisternally leave the inoculated animals essentially unchanged so far as response to more potent or larger doses of virus is concerned.

Finally, observations on attempted active immunization through inoculation of the mucous membranes of the upper respiratory tract will be recorded. Experiments of this sort, on a wider scale, would seem to be called for in view of the common belief that it is by means of a subclinical process of inoculation of the nasal and adjacent mucosa in man that the observed immunity of adult populations is taking place. Besides lower monkeys, these tests should perhaps be made also on higher apes.

---

* Presented before the Association of American Physicians, Atlantic City, N. J., May 3, 1932.

Human and Monkey Passage Virus Immunity

There are very few references in the literature which may be taken to indicate infective or antigenic distinctions in the virus of poliomyelitis. Common experience has shown that the virus of American and of European poliomyelitis can be neutralized with human convalescent sera. While specimens of the virus are known to fluctuate in virulence, and the convalescent and other neutralizing sera to vary in antiviral strength, no experimental evidence that these differences represent more than quantitative distinctions has been offered until recently. Burnet and Macnamara (3) drew attention to a variation between virus obtained in an epidemic of poliomyelitis in Victoria and the standard Rockefeller Institute specimen, and Weyer (4) described quantitative differences in the neutralizing value of convalescent and antiviral horse serum for recent (1931) and monkey passage (Aycock) virus strains.

The 1931 epidemic of poliomyelitis provided many human specimens of spinal cord and medulla. These were collected at autopsies, placed into 50 per cent chemically pure glycerol, and kept at +4°C. In due time, monkeys (*Macacus rhesus*) were inoculated in order to establish the virus as passage strains, and in addition a group of five monkeys was given several intradermal injections of the human material, not passed through monkeys, by the method of Aycock and Kagan (5). Four of these animals showed no symptoms of disease, while the fifth became definitely paralyzed in one leg. Recovery of this animal occurred with residual atrophy and partial paralysis of the leg muscles.

Two sets of experiments were made with these monkeys. Blood was taken from all five to be tested for antiviral power. Two of the four symptomless animals were given 7 daily instillations of the Rockefeller Institute monkey passage virus. These monkeys developed paralytic poliomyelitis to which they succumbed in the same manner and time as did the control animal. The sera of the symptom-free as well as of the paralyzed and recovered monkeys were next tested for antiviral action against a Berkefeld filtrate of the monkey passage virus. No neutralization, in the dilutions employed, took place. These dilutions were such as to develop the antiviral property in sera from monkeys immunized with the passage strain.

Three monkeys of this series still survive and are to be tested for resistance and serum antiviral power against the 1931 virus implanted in monkeys with as few passages as possible.[1]

Monkeys were actively immunized by the intradermal method of inoculation with the first monkey passage of the 1931 virus. The blood serum failed to neutralize the monkey passage strain, and the serum of one monkey of this series, which developed and recovered from an abortive infection, was devoid of antiviral properties for this strain. The converse of these tests was carried out with the 1931 virus in its fifth monkey passage with human convalescent and monkey passage reinforced (hyperimmune) sera. Both proved neutralizing. This result was perhaps predictable in view of the common observation that human convalescent serum is antiviral for recent and remote monkey passage strains. Now that the 1931 virus appears to have become adapted to monkeys, these experiments can be extended so as to provide information bearing on the qualitative and quantitative relationships of the monkey passage virus which goes back, in part, to the 1909 epidemic of poliomyelitis, and the 1931 strain. A comparison will also be possible with the Victorian virus, now passed through a number of monkeys, kindly sent us by Drs. Burnet and Macnamara.

---

[1] Difficulties have been encountered in establishing the 1931 human virus in *Macacus rhesus* monkeys. It is common experience that a part only of human specimens can be successfully and enduringly implanted in monkeys. The reasons for the refractoriness of the 1931 virus in this respect are not known. Recently we have secured a specimen of virus which in the fifth and sixth passages is effective and, curiously enough, has induced prostrating paralysis in two of three monkeys on the third intradermal inoculation carried out for purposes of active immunization.

It is not known whether the modified passage virus has lower infective power for man than has the original human virus. Undoubtedly the effective virus is changed in this respect through adaptation to monkeys, since so many failures occur in this process. No instance of laboratory infection in man has come to our attention. An experiment is under way with the chimpanzee in order to ascertain whether this species, more nearly related biologically to man, responds in an unusual manner to the passage virus. If peculiarities should appear, a corresponding test can be made with the 1931 virus material.[2]

Virus-Serum Immunity

The common method of inducing active immunity in monkeys is by repeated subcutaneous or intradermal injections of virus (6). The injections are made once or twice a week over a period of several weeks. A rest period of from 4 to 8 weeks is given, after which the tests for immunity are performed. These tests consist of serum neutralization determinations and intracerebral or intracisternal virus injections. It happens, at times, that a serum proves to be antiviral while the animal yielding it develops paralysis. In other words, the degree of immunity is relative. The existence of an immunity is more delicately exhibited by the neutralization than by the severer infection test. Since normal monkey serum is consistently devoid of antiviral action, the neutralization definitely indicates a state of immunity.

Monkeys and human beings that have passed through and recovered from poliomyelitis yield antiviral sera (7). Convalescent monkey serum is weaker in neutralizing action than convalescent human serum. But the strength of the monkey serum can be increased and brought to a high titre by subsequent subcutaneous virus inoculations. This process is called reinforcement (8).

Active immunity in the monkey, experimentally inoculated, is therefore a relative state. Even with repeated intradermal injections of virus, occasional monkeys give only low immunity responses. The comparative immunity value of the abortive as compared with the frankly paralytic experimental infection has been little studied. We have compared recently two *Macacus rhesus* monkeys in which, as a result of the injection of the same virus, one developed merely tremor and ataxia and the other paralysis of arms and legs from which partial recovery took place. These animals were tested subsequently by intracerebral inoculation and serum neutralization. The paralyzed monkey withstood an inoculation to which the slighter (abortive) affected animal succumbed, and the serum of the former possessed higher antiviral power than that of the latter.

---

[2] A female chimpanzee weighing 70 pounds was given an intracerebral inoculation of a potent Berkefeld filtrate of the monkey passage virus. No symptoms having developed, a second or accelerating (1, 13) injection was given 10 days later. This animal has resisted both injections.

Monkeys given several subcutaneous or intradermal injections of virus sometimes develop paralysis instead of immunity. The proportion of those becoming paralyzed has varied with different experimenters (6). There has been too little consistency in the various tests to make comparison accurate. The strain or sample of virus undoubtedly plays a part, some being more virulent than others. Dosage also plays a part. Because of this disparity, immunity has been sought by means of fewer injections and the combined employment of immune serum and virus. A substitution of one or few larger injections of virus, or of virus and serum, has been made for the repeated injections (9).

Undoubtedly active immunity can be produced in this manner. A closer study of the process is called for. The experiments so far reported relate to few animals, or are so varied as to be inconclusive (10). The fact remains, however, that mixtures of virus and serum, probably underneutralized, and separately and simultaneously injected virus and serum, give rise to some degree of active immunity (11). A question which the inadequate experiments have not answered is whether, when virus and serum are simultaneously injected, symptoms of infection ever arise. We have made one separate injection of virus intradermally and of serum subcutaneously in fourteen *Macacus rhesus* monkeys without eliciting symptomatic effects in any. From 0.5 to 1.0 gm. of potent monkey passage virus and varying quantities of reinforced monkey or human convalescent serum were given on the same day. That active immunity develops under these conditions can be shown by neutralization and infection tests. The grade of immunity thus produced remains to be determined, as well as the optimum conditions under which it arises without, at the same time, being attended by abortive or paralytic symptoms of disease.

We are now better informed as to the qualitative and quantitative nature of active immunity produced in monkeys. It is not sufficient merely to test the animals with virus on the basis of "virulence." Account should also be taken of the effect of the strain, that is, whether the strain is homologous or heterologous. The results supposed to have been attained may well be changed by this circumstance. In this connection reference may be made to the report of Stewart and Rhoads (6) in which the actively immunized monkeys responded differently according as the Rockefeller Institute, M.A. or Aycock strains of virus were employed for the immunizing and for the test inoculations.

No convincing evidence exists that the virus of poliomyelitis is qualitatively modified by chemical or physical agents. All the effects ascribed to these agents may be due to quantitative changes only. The viruses, apparently weakened by chemicals and heat or drying, when still effective, regain their lost potency quickly on monkey passage. A state of prolonged but not permanent depression sometimes occurs, which is not to be explained in this way, and has been related to the wave movements of epidemic poliomyelitis (12). The latter phenomenon calls for renewed study in the light of fuller knowledge of the biology of the virus.

Nasal Instillation and Immunity

Reference should be made to the effect of nasal instillation of the virus in monkeys. Paralytic poliomyelitis is readily induced in this manner by from 3 to 6 or 7 daily instillations. The number of refractory animals is not large. Several such resistant animals were assembled and subjected to repeated nasal instillations with intervals of one or more weeks between the courses. The refractory monkeys, which resisted not only one but several courses of instillation, were tested for active immunity by (*a*) serum neutralization, and (*b*) intracerebral inoculation. No indication of an immunity was secured.

On the other hand, one nasally instilled monkey which, from a course of instillations, developed fleeting, abortive, non-paralytic symptoms, yielded an antiviral serum and resisted the intracerebral injection.

These experiments should be carried further and on a larger scale in order to determine whether the monkey, naturally not affected by epidemic poliomyelitis but experimentally susceptible, is capable of being immunized, as man is supposed to be, by way of the mucous membranes of the upper respiratory tract, in the absence of the appearance of all symptoms of disease.

SUMMARY

1. The passage of the virus of poliomyelitis through monkeys intensifies its infectivity for these animals and modifies its specific immunologic reactions. The changes undergone are quantitative and probably also qualitative in nature. The modified virus preserves its immunizing power, in part at least, against recent strains of the human virus. Immune sera prepared with human and with monkey passage strains of virus exhibit quantitative differences in cross-neutralizing tests.
2. Virus-serum immunity can be produced in monkeys by the separate, simultaneous injection of one or more doses of virus and immune serum. The dangers of active poliomyelitic symptoms arising in the inoculated animals seem lessened by the combined treatment. The optimum manner of producing virus-serum immunity has still to be determined.
3. Monkeys that have proved wholly refractory to nasal instillations of the virus have been shown to be devoid of serum antiviral activity and to exhibit average susceptibility to intracerebral inoculation of potent virus.

REFERENCES

1. Flexner, Simon, and Lewis, P. A., *J. Am. Med. Assn.*, 1910, **54**, 45, and 1780; *J. Exp. Med.*, 1910, **12**, 227.
   Flexner, Simon, *J. Am. Med. Assn.*, 1910, **55**, 1105.
2. The complete paper will appear in the *J. Exp. Med.*
3. Burnet, F. M., and Macnamara, J., *Brit. J. Exp. Path.*, 1931, **12**, 57.
4. Weyer, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1931, **29**, 289.
5. Aycock, W. L., and Kagan, J. R., *J. Immunol.*, 1927, **14**, 85.
6. Flexner, Simon, and Lewis, P. A., *J. Am. Med. Assn.*, 1910, **55**, 662.
   Stewart, F. W., and Rhoads, C. P., *J. Exp. Med.*, 1929, **49**, 959.
   Aycock, W. L., and Kagan, J. R., *J. Immunol.*, 1927, **14**, 85.

7. Römer, P. H., and Joseph, K., *Münch. med. Woch.*, 1910, **57**, 568.
Levaditi, C., and Landsteiner, K., *Compt. rend. Soc. biol.*, 1910, **68**, 311.
Flexner, Simon, and Lewis, P. A., *J. Am. Med. Assn.*, 1910, **54**, 1780.
Netter, A., and Levaditi, C., *Presse méd.*, 1910, **18**, 268.
8. Flexner, Simon, and Lewis, P. A., *J. Am. Med. Assn.*, 1910, **54**, 1780.
Flexner, Simon, *J. Am. Med. Assn.*, 1910, **55**, 1105.
Rhoads, C. P., *J. Exp. Med.*, 1931, **53**, 137.
9. Flexner, Simon, and Lewis, P. A., *J. Am. Med. Assn.*, 1910, **55**, 662.
Rhoads, C. P., *J. Exp. Med.*, 1930, **51**, 1; 1931, **53**, 115.
10. Brodie, M., and Goldbloom, A., *J. Exp. Med.*, 1931, **53**, 885.
11. Rhoads, C. P., *J. Exp. Med.*, 1931, **53**, 115.
Brodie, M., and Goldbloom, A., *J. Exp. Med.*, 1931, **53**, 885.
12. Flexner, Simon, Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, **19**, 195.
Levaditi, C., and Hornus, G., *Bull. Acad. méd.*, Paris, 1932, **107**, series 3, 580.
13. Flexner, Simon, *Science*, 1931, **74**, 520.

[Reprinted from The Journal of Biological Chemistry, September, 1932,
Vol. 97, No. 3, pp. 717-726]

THE RELATION OF CHEMICAL STRUCTURE TO THE RATE OF HYDROLYSIS OF PEPTIDES

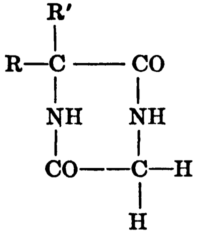
VII. Hydrolysis of Dipeptides by Alkali

By P. A. LEVENE, ROBERT E. STEIGER, and ALEXANDRE ROTHEN
(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, July 6, 1932)

Great differences have been observed in the rates of hydrolysis of dipeptides as well as in those of ketopiperazines in alkaline medium.[1,2] Glycyl-glycine and glycyl-glycine anhydride, the simplest representatives of these two types of compounds, are much more rapidly hydrolyzed than any of their respective derivatives obtained by substitution of the hydrogen atoms of the methylene groups with alkyl or aryl groups.

Measurements, as effected on a series of ketopiperazines of the type represented in I, have shown[2] that the stability of the ring structure varies greatly with the nature of the radicles R and R′ attached to the nucleus. The names of some of the anhydrides



I

---

[1] Levene, P. A., Bass, L. W., and Steiger, R. E., *J. Biol. Chem.*, **82**, 167 (1929).

[2] Levene, P. A., Rothen, A., Steiger, R. E., and Osaki, M., *J. Biol. Chem.*, **86**, 723 (1930).

studied, arranged in the order of decreasing rates of hydrolysis or of increasing ring stability, are given in Table I. In these experiments 5 mols of sodium hydroxide had been allowed to act upon 1 mol of ketopiperazine, the initial concentration of sodium hydroxide being 0.5 N and the temperature 25°.

In an earlier paper[1] the hydrolysis of several dipeptides by alkali had been studied. The experimental conditions were the same as those above. Both the rates of hydrolysis of glycyl-alanine and of alanyl-glycine were found to be considerably lower than that of glycyl-glycine. Glycyl-α-aminoisobutyric acid was not hydrolyzed by the alkali of the concentration used.

TABLE I

Ketopiperazines Arranged in Order of Increasing Ring Stability

Glycyl-glycine anhydride (symmetric)
Alanyl-glycine (or glycyl-alanine) anhydride
Leucyl-glycine (or glycyl-leucine) anhydride
Valyl-glycine (or glycyl-valine) anhydride
α-Aminoisobutyryl-glycine (or glycyl-α-aminoisobutyric acid) anhydride

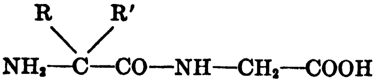
TABLE II

Hydrolysis Constants of Peptides

Peptides, Series A	$k \cdot 10^3$	Peptides, Series B	$k \cdot 10^3$
Glycyl-glycine	152*	Glycyl-glycine	152*
Alanyl-glycine	32*	Glycyl-alanine	37*
Leucyl-glycine	7.3	Glycyl-leucine	9.3
Valyl-glycine	0	Glycyl-valine	7.5
α-Aminoisobutyryl-glycine†		Glycyl-α-aminoisobutyric acid	0*

* Figures previously reported.
† Not available for investigation.

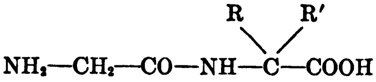
The following dipeptides, *d,l*-leucyl-glycine, *d,l*-valyl-glycine, glycyl-*d,l*-leucine, and glycyl-*d,l*-valine, have since been prepared and submitted to the action of alkali under the conditions adopted in all our previous work. Their hydrolysis constants, together with those of the peptides mentioned in the preceding paragraph, are given in Table II. Peptides of Series A are derived from glycyl-glycine by substitution on the methylene group of the chain carrying the amino group (see II). Peptides of Series B are



$$\mathrm{NH_2{-}\overset{R\quad R'}{\overset{\diagdown\ \diagup}{C}}{-}CO{-}NH{-}CH_2{-}COOH}$$

II

derived from glycyl-glycine by substitution on the methylene group of the chain carrying the carboxyl group (see III). Members



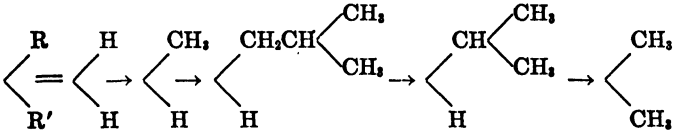
$$\mathrm{NH_2{-}CH_2{-}CO{-}NH{-}\overset{R\quad R'}{\overset{\diagdown\ \diagup}{C}}{-}COOH}$$

III

of each series are named in the order of decreasing rates of hydrolysis or increasing stability.

An interesting parallelism in the behavior towards alkali of dipeptides and of ketopiperazines becomes apparent from a comparison of the contents of Tables II and I: *any two radicles, R and R′, have the same relative effect on the stability of the peptide linkage as on the stability of the ring structure.*

When classified according to increasing stabilizing effects, these radicles appear for all the series of compounds considered in the following order.



The experimental data are recorded in Table III. This also contains hydrolysis figures obtained anew for the dipeptide alanyl-alanine, already studied some years ago.[1] The titration values found at that time for this compound were somewhat irregular and it has since been felt that the experiment should be repeated. As a result, the hydrolysis constant $k \cdot 10^3 = 6.5$ (previously found = 7.6) has been obtained. Finally, figures are presented showing that the dipeptide leucyl-leucine is resistant to the action of the alkali of the concentration used.

Thus, both glycyl-α-aminoisobutyric acid ($k \cdot 10^3 = 0$) and alanyl-alanine ($k \cdot 10^3 = 6.5$) are more stable than alanyl-glycine ($k \cdot 10^3 = 32$) or glycyl-alanine ($k \cdot 10^3 = 37$), while leucyl-leucine ($k \cdot 10^3 = 0$) is more stable than leucyl-glycine ($k \cdot 10^3 = 7.3$) or glycyl-leucine ($k \cdot 10^3 = 9.3$). It is seen from the preceding that *the stability of the peptide linkage is enhanced by the introduction in glycyl-glycine of a substituent R and still further, by the introduction of a second group R of the same nature.*

It can be seen from Table II that the rates of hydrolysis of the peptides of Series B are throughout higher than those of the corresponding isomers of the Series A. *If, into glycyl-glycine a group R be introduced, it stabilizes the peptide linkage to a greater extent when adjacent to the amino group than when adjacent to the carboxyl.*

The comparison of the hydrolysis constants of the two isomers glycyl-α-aminoisobutyric acid ($k \cdot 10^3 = 0$) and alanyl-alanine ($k \cdot 10^3 = 6.5$) shows that *two identical groups R, introduced into glycyl-glycine, stabilize the peptide linkage to a greater extent when attached to the same carbon atom than when distributed over both methylene groups of the chain.*

EXPERIMENTAL

1. Preparation of Dipeptides

d,l-Valyl-Glycine

(*a*) *d,l-Bromoisovaleryl-Glycine*—This compound has already been obtained by Fischer and Schenkel.[3] Abderhalden, Sah, and Schwab[4] followed Fischer and Schenkel's indications. The procedure described has been modified as follows: A solution of 15 gm. (0.2 mol) of glycine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was cooled in an ice-water mixture during treatment with 40 gm. (0.2 mol) of *d,l*-α-bromoisovaleryl chloride and 100 cc. of 2.0 N sodium hydroxide, these reagents being added in equivalent proportions with continuous shaking. The crude bromo compound precipitated as a solid on the addition of 52 cc. of 5.0 N hydrochloric acid (0.26 mol). It weighed dry, 42 gm. (yield, 88 per cent of the theory).

(*b*) *d,l-Valyl-Glycine*—This peptide was obtained for the first time by Fischer and Schenkel.[3] These authors, having observed that the amination of the above bromo compound proceeded very slowly at room temperature, chose to aminate at 100°. The yield of crude product was 35 per cent of the theory. Secondary products, among which was an unsaturated one (presumably dimethylacrylyl-glycine), were formed in large amounts. Abderhalden, Sah, and Schwab[4] modified this procedure by aminating at 37° (4 days). Their yield was nearly the same as that of Fischer and Schenkel. They confirmed these authors' observations as to the formation of alcohol-soluble by-products. However, we have found that when the amination is carried out at room temperature in the way described below, the yield of dipeptide is the same as that already recorded and that the alcohol-soluble fraction then consists of the ammonium salt of unchanged *d,l*-bromoisovaleryl-glycine. 42 gm. of crude *d,l*-α-bromoisovaleryl-glycine were allowed to stand for 10 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was diluted with an equal volume of water and concentrated to dryness under reduced pressure. The crystalline residue was dissolved in 100 cc. of water and the solution was evaporated to dryness. This operation was repeated. The residue was then evaporated to dryness in the presence of 100 cc. of absolute alcohol. In order to remove the water completely, this was repeated four times. The final residue, a mixture of *d,l*-valyl-glycine and of the ammonium salt of unchanged *d,l*-α-bromoisovaleryl-glycine, was extracted with boiling absolute alcohol. The mixture was filtered while hot. The dipeptide which had remained undissolved was washed with hot absolute alcohol. It weighed 9 gm.

---

[3] Fischer, E., and Schenkel, J., *Ann. Chem.*, **354,** 13, 14 (1907).

[4] Abderhalden, E., Sah, P., and Schwab, E., *Fermentforschung*, **10,** 265 (1928–29).

The alcoholic extract gave, on cooling, a crystalline precipitate of the *ammonium salt of d,l-α-bromoisovaleryl-glycine*. This was filtered off, washed with cold absolute alcohol, and air-dried. It weighed 13 gm. The filtrate was discarded. The suspension of this material in 35 cc. of water was treated with 14.5 cc. of 5.0 N hydrochloric acid, whereupon the free bromo acid precipitated. It was washed with water and air-dried. It weighed 10.9 gm. The *d,l-α-bromoisovaleryl-glycine* was crystallized from boiling benzene. It melted at 139–140° (uncorrected). Fischer and Schenkel[3] found 139–141° (corrected).

No. 190. 5.316 mg. substance: 6.914 mg. CO_2 and 2.405 mg. H_2O
0.1000 gm. " : 4.33 cc. 0.1 N HCl (Kjeldahl)
0.1168 " " : 0.0907 gm. AgBr (Carius)

$C_7H_{12}O_3NBr$ (238.03). Calculated. C 35.29, H 5.08, N 5.89, Br 33.58
Found. " 35.46, " 5.06, " 6.06, " 33.04

The crude *d,l*-valyl-glycine was dissolved in about 30 cc. of water in the presence of some norit. 400 cc. of hot absolute alcohol were added to the filtrate. Crystallization set in very soon; the mixture was cooled in ice and water. 5.6 gm. of dipeptide were obtained. They were dissolved in 26 cc. of water in the presence of some norit; 400 cc. of hot absolute alcohol were added to the filtrate. Crystallization took place immediately. The mixture was allowed to stand overnight in ice and water. The crystals were washed with alcohol. Yield, 4.4 gm.

No. 198. 5.855 mg. substance: 10.420 mg. CO_2 and 4.490 mg. H_2O
6.240 " " : 0.867 cc. N (23°, 757 mm.)
$C_7H_{14}O_3N_2$ (174.13). Calculated. C 48.24, H 8.10, N 16.09
Found. " 48.53, " 8.58, " 15.96
Moisture, none

d,l-Leucyl-Glycine

(*a*) *d,l-α-Bromoisocapronyl-Glycine*—The preparation was carried out in a manner similar to that for *d,l-α*-bromoisovalerylglycine.

(*b*) *d,l-Leucyl-Glycine*[5]—49 gm. of crude *d,l-α*-bromoisocapronyl-glycine were allowed to stand for 3 days at room temperature with 10 times this weight of ammonium hydroxide (sp. gr. 0.90). The solution was then diluted with water, clarified with norit, filtered, and evaporated to dryness. The residue was dissolved in about 16 parts of boiling water. The solution was clarified with norit, then 1100 cc. of absolute alcohol were added. Yield, 26 gm. 23.8 gm. of this material were dissolved in 16 parts of boiling water, 1100 cc. of absolute alcohol were added to the clear solution, and the mixture was left to stand in the cold room. The peptide was washed with absolute alcohol. It weighed dry, 19.6 gm.

No. 260. 5.195 mg. substance: 9.720 mg. CO_2 and 3.845 mg. H_2O
0.1000 gm. " : 10.55 cc. 0.1 N HCl (Kjeldahl)
$C_8H_{16}O_3N_2$ (188.15). Calculated. C 51.03, H 8.57, N 14.89
Found. " 51.04, " 8.28, " 14.77
Moisture, none

---

[5] *Cf.* Fischer, E., *Ann. Chem.*, **340**, 144 (1905).

Glycyl-d,l-Valine

(*a*) *Chloroacetyl-d,l-Valine*—The details of the preparation of this compound were worked out 3 years ago. Meanwhile there appeared a paper by Abderhalden, Rindtorff, and Schmitz in which a method for preparing this compound is described.[6] The procedure given below is simpler. A solution of 23.5 gm. (0.2 mol) of *d,l*-valine in 100 cc. of 2.0 N sodium hydroxide (0.2 mol) was cooled in an ice-water mixture during treatment with 46 gm. (0.4 mol) of chloroacetyl chloride and 100 cc. of 5.0 N sodium hydroxide (0.5 mol), these reagents being added in equivalent proportions with continuous shaking. The chloroacetyl compound precipitated on addition of 72 cc. of 5.0 N hydrochloric acid (0.36 mol). It was washed with ice water, then with petrolic ether, and dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It weighed dry, 32 gm. (yield, 82 per cent of the theory).

(*b*) *Glycyl-d,l-Valine*—This compound was obtained in a somewhat different way from that described by Abderhalden, Rindtorff, and Schmitz.[6] Our procedure was as follows: A mixture of 32 gm. of crude chloroacetyl-*d,l*-valine with 10 times this weight of ammonium hydroxide (sp. gr. 0.90) was kept at room temperature for 3 days, during which time it was occasionally shaken. The solution was diluted with water, clarified with norit, and concentrated to dryness under reduced pressure. The residue was dissolved in 60 cc. of water. Norit was added. 700 cc. of absolute alcohol were added to the filtrate; the solution was allowed to cool in the ice box. Additional 300 cc. of alcohol were added to the mixture. The crystals were then filtered off and washed thoroughly with hot absolute alcohol. Yield, 23.5 gm. This product contained traces of chlorine and ammonia. It was dissolved in 70 cc. of hot water; norit and, after filtration, 500 cc. of absolute alcohol were added to the solution. The mixture obtained was cooled overnight in ice water. The crystals were washed with hot absolute alcohol and then dried in a desiccator over phosphorus pentoxide. Yield, 21 gm. As this material still contained 7.1 per cent of moisture, it was heated in a vacuum oven to 70°. For analysis the substance was redried at 100° under diminished pressure.

---

[6] Abderhalden, E., Rindtorff, E., and Schmitz, A., *Fermentforschung*, **10**, 220 (1928–29).

No. 182. 3.955 mg. substance: 6.990 mg. CO_2 and 2.845 mg. H_2O
4.105 " " : 0.583 cc. N (20°, 735 mm.)
$C_7H_{14}O_3N_2$ (174.13). Calculated. C 48.24, H 8.10, N 16.09
Found. " 48.19, " 8.05, " 16.00
Moisture, 0.41 per cent

Glycyl-d,l-Leucine

(*a*) *Chloroacetyl-d,l-Leucine*—The preparation was carried out in a similar manner to that of chloroacetyl-*d*,*l*-valine.

(*b*) *Glycyl-d,l-Leucine*—Fischer[7] obtained this dipeptide by heating a mixture of 1 part of chloroacetyl-*d*,*l*-leucine and 5 parts of ammonium hydroxide to 100°. This procedure was not followed. Instead, the crude chloroacetyl-*d*,*l*-leucine was allowed to stand for 3 days at room temperature with 10 times its weight of ammonium hydroxide (sp. gr. 0.90). The peptide formed was then isolated and purified in the usual way.

No. 261. 3.900 mg. substance: 7.330 mg. CO_2 and 3.105 mg. H_2O
0.1000 gm. " : 10.48 cc. 0.1 N HCl (Kjeldahl)
$C_8H_{16}O_3N_2$ (188.15). Calculated. C 51.03, H 8.57, N 14.89
Found. " 51.25, " 8.90, " 14.67
Moisture, none

Dextro-Alanyl-Dextro-Alanine

Sample 267 of this substance, described in a preceding publication,[8] was used.

Dextro-Leucyl-Dextro-Leucine

Sample 263 of this substance described in a preceding publication,[8] was used.

2. Procedure in Hydrolysis Experiments

5 mols of sodium hydroxide were allowed to act upon 1 mol of dipeptide, the concentration of sodium hydroxide being 0.5 N and the temperature 25°. The procedure used for each peptide was the same as that described in a previous publication.[1]

[7] Fischer, E., *Ann. Chem.*, **340**, 157 (1905).

[8] Levene, P. A., Steiger, R. E., and Marker, R. E., *J. Biol. Chem.*, **93**, 605 (1931).

TABLE III

*Hydrolysis Constants of Dipeptides**

d,l-Leucyl-glycine			*d,l*-Valyl-glycine			Glycyl-*d,l*-leucine			Glycyl-*d,l*-valine					Dextro-alanyl-dextro-alanine					Dextro-leucyl-dextro-leucine		
t	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$	*t*	*V*	*t*	*V*	$k \cdot 10^3$	*t*	*V*	*t*	*V*	$k \cdot 10^3$	*t*	*V*	$k \cdot 10^3$
days	*cc.*		*days*	*cc.*		*days*	*cc.*		*days*	*cc.*	*days*	*cc.*		*days*	*cc.*	*days*	*cc.*		*days*	*cc.*	
0	2.54		0	2.53_5		0	2.53		0	2.53	0	2.51		0	2.53	0	2.52		0	2.54	
1.08	2.61	(11.4)	3	2.56_5	0	2	2.66	(11.6)	5.67	2.80			(8.8)			7.8	2.80	6.6	3	2.54	0
6	2.78	7.3	6	2.56	0	4.17	2.75	9.6			5.92	2.79	(8.7)	11	2.92			6.7	9	2.58	0
11	2.97	7.5	9	2.56_5	0	9.08	2.98_5	9.6			7.92	2.86_5	(8.4)			13	3.00	7.1	13	2.56_5	0
18	3.19	7.3	13	2.55	0	10.96	3.07	9.6	10.83	2.96_5			7.7	15	3.03			6.5	18	2.58	0
23	3.34	7.3	18	2.57	0	14	3.19	9.5			13.92	3.07	7.9			18	3.11	6.5	25	2.57	0
28	3.47	7.2	25	2.57	0	17	3.30_5	9.5	15.92	3.15			7.8	21	3.21			6.6	32	2.58	0
33	3.58_5	7.1	32	2.58	0	19	3.37	9.4			20.92	3.28	7.6			23	3.26	6.6			
37	3.71	7.4				24	3.52	9.1	22.88	3.33			7.3	28	3.36			6.3			
						26	3.57	8.7			25.92	3.41	7.5			30	3.41	6.4			
						29	3.66	9.0	29.88	3.51			7.2	33	3.47			6.2			
						33	3.74	8.7	33.88	3.59			7.1			37	3.57	6.4			
Average..		7.3			0			9.3					7.5					6.5			0

* Values of $k \cdot 10^3$ in parentheses have not been included in calculating average values. $k \cdot 10^3$ is calculated for days.

The rates of hydrolysis were calculated with the following equation

$$k \cdot t = \log_{10} \frac{a}{a - x}$$

The results are given in Table III.

It may be said that not too great an importance should be attributed to the decimals given for $k \cdot 10^3$. The procedure used was not sufficiently precise to permit, for instance, of a definite statement to the effect that leucyl-glycine ($k \cdot 10^3 = 7.3$) is less rapidly hydrolyzed than glycyl-valine ($k \cdot 10^3 = 7.5$).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, October, 1932,
Vol. 98, No. 1, pp. 321–332]

STUDIES ON RACEMIZATION

XI. ACTION OF ALKALI ON POLYPEPTIDES

BY P. A. LEVENE AND ROBERT E. STEIGER

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, July 28, 1932)

In a previous publication[1] a report was made on the behavior towards alkali of di- and tripeptides entirely composed of optically active amino acids. It had been established that the dipeptide dextro-leucyl-dextro-leucine, when submitted to the action of 10 equivalents of 1.0 N sodium hydroxide at 25° over a prolonged period of time (8 days), did not suffer any racemization, whereas the tripeptides levo-alanyl-dextro-leucyl-dextro-leucine and dextro-leucyl-dextro-leucyl-dextro-leucine, when subjected to the same treatment, were partly racemized. The conclusion was drawn that migration, under the influence of alkali, of the hydrogen atoms attached to asymmetric carbon atoms takes place only on the central amino acids of a polypeptide chain, the terminal amino acids which carry the free amino or the carboxyl group remaining wholly unaltered. The prediction of Dakin[2] that racemization in polypeptides would take place only in systems composed of at least three amino acids was thus substantiated.

No loss of optical activity having been observed when the two tripeptides mentioned above were treated with alkali of lower concentration (2 equivalents of 0.2 N sodium hydroxide), an additional conclusion was reached; namely, that a minimum concentration of alkali was required to produce the significant change to some appreciable degree. Observations in this direction had already been made by Levene and Pfaltz[3] with polypeptides composed of levo-alanine and glycine. They had observed that the racemization increased with the concentration of the sodium hydroxide solutions used. These, however, (and sometimes the temperatures) were kept low because of the simultaneous, quite rapid hydrolysis of the peptides under the influence of the alkali. The leucine tripeptides studied later were highly resistant to the action of even 1.0 N sodium hydroxide; in fact, they were selected for study for precisely that reason. With little or no hydrolysis to the optically stable dipeptide taking place, it seemed that these compounds—or better, their central amino acid unit—should be racemized in alkali of such high concentration to a much greater extent than was actually the case. The question naturally arises whether the racemization of any central amino acid unit of a polypeptide chain varies greatly with the nature of the alkyl (or aryl) group attached to the asymmetric carbon atom under consideration. An answer to this may be found in a forthcoming publication.

---

[1] Levene, P. A., Steiger, R. E., and Marker, R. E., *J. Biol. Chem.*, **93**, 605 (1931).

[2] Dakin, H. D., *J. Biol. Chem.*, **13**, 362 (1912–13).

[3] Levene, P. A., and Pfaltz, M. H., *J. Gen. Physiol.*, **8**, 183 (1925); *J. Biol. Chem.*, **68**, 277 (1926); **70**, 219 (1926).

The present work was undertaken in order to see to what extent the degree of racemization of such an amino acid (*e.g.*, dextro-leucine) is dependent on the nature of the adjacent or all other amino acid units of the molecule or varies with the position occupied by this amino acid within the chain. Two tetrapeptides (of fair resistance to the hydrolytic action of 1.0 N sodium hydroxide), just obtained, have now been examined with regard to their optical stability under the conditions specified.

The results are given in Table I. The preparation of the tetrapeptides and of the intermediates is described in the experimental part. The data of the racemization and the control hydrolysis experiments are recorded in Tables II and III.

It can be seen from Table I that there is considerable loss of optical activity when the leucyl unit of the tripeptide dextro-leucyl-dextro-leucyl-dextro-leucine, carrying the carboxyl group, is replaced by glycyl-glycine. For the tetrapeptide glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine, which contains two leucine units susceptible to racemization, the loss of optical activity was higher than that observed either for the parent substance or for the preceding compound. It must be borne in mind, however, that the figures given in Table I do not represent the racemization values of the central leucine units of the peptides, since in the calculations no allowance has been made for the terminal asymmetric amino acids which, as has been shown, retain their optical activity.

EXPERIMENTAL

1. *Preparation of Compounds*

Levo-α-Bromoisocapronyl-Glycyl-Glycine

46 gm. (0.4 mol) of finely powdered glycine anhydride[4] and 400 cc. of 1.0 N sodium hydroxide (0.4 mol) were placed in a well stoppered bottle and the mixture was shaken at room temperature until a clear solution was obtained (hydrolysis to the dipeptide). This was then cooled in an ice and water mixture during treatment with 86 gm. (0.4 mol) of levo-α-bromoisocapronyl chloride and 400 cc. of 1.0 N sodium hydroxide (0.4 mol), these reagents being added in equivalent proportions with continuous shaking. 104 cc. of 5.0 N hydrochloric acid (0.52 mol) were then added and a crystalline precipitate of the bromo compound was obtained at once. This was filtered off, washed with ice water, then with petrolic ether, and was dried in a vacuum desiccator over phosphorus pentoxide. Yield, 99.0 gm. (80 per cent of the theory). This material was dissolved in 693 gm. of absolute ethyl acetate (7 parts by weight) at boiling temperature. Norit was added and the

TABLE I

Action on Peptides of 1.0 N Sodium Hydroxide at 25°

Compound	Loss of optical activity after 8 days with 10 mols 1.0 N NaOH at 25°
	per cent
d-Leucyl-*d*-leucine	0*
l-Alanyl-*d*-leucyl-*d*-leucine	5*
d-Leucyl-*d*-leucyl-*d*-leucine	4*
d-Leucyl-*d*-leucyl-glycyl-glycine	19
Glycyl-*d*-leucyl-*d*-leucyl-*d*-leucine	23

* Figures previously reported.

---

[4] Prepared from glycine ethyl ester hydrochloride in the way indicated by Fischer, E., *Ber. chem. Ges.*, 39, 2930 (1906).

solution was filtered while hot. Crystallization set in immediately. The crystals were filtered off at room temperature and were well washed with ethyl acetate. They were dried in a vacuum oven at 50° until free of solvent. They weighed 87.0 gm. For analysis and rotation a fraction of this material was recrystallized twice from boiling absolute ethyl acetate. It then sintered and melted between 130–132° (uncorrected).

No. 493. 4.545 mg. substance: 6.540 mg. CO_2 and 2.255 mg. H_2O
5.455 " " : 0.445 cc. N (24°, 756.7 mm.)
6.000 " " : 3.625 mg. AgBr (Carius)
$C_{10}H_{17}O_4N_2Br$ (309.08). Calculated. C 38.83, H 5.55, N 9.06, Br 25.86
Found. " 39.23, " 5.55, " 9.34, " 25.70

It had the following rotations.

$$[\alpha]_D^{20} = \frac{-5.93° \times 100}{4 \times 3.0824} = -48.1° \text{ (in absolute alcohol)}$$

0.4636 gm. in 15.04 cc. of solution (0.1 M)

$$[\alpha]_D^{20} = \frac{-2.25° \times 100}{4 \times 1.5453} = -36.4° \text{ (in water)}$$

0.2318 gm. in 15.00 cc. of solution (0.05 M)

Abderhalden and Fodor[5] found for dextro-α-bromoisocapronyl-glycyl-glycine $\alpha_D^{20} = +0.83°$ (0.5 dm. tube) or $[\alpha]_D^{20} = +47.01°$, employing a solution of 0.2789 gm. in absolute alcohol, the weight of the solution being 6.3659 gm. They found $\alpha_D^{20} = +0.23°$ (0.5 dm. tube) or $[\alpha]_D^{20} = +31.17°$, employing a solution of 0.2250 gm. in water, the weight of the solution being 15.4591 gm. Their material melted at 130–132°.

Dextro-Leucyl-Glycyl-Glycine

A solution of 81 gm. of levo-α-bromoisocapronyl-glycyl-glycine (crystallized once from ethyl acetate) in 810 gm. of ammonium hydroxide of sp. gr. 0.90 was kept at room temperature for 3 days. It was then diluted with water and concentrated to a small volume under reduced pressure. Water was then repeatedly added and evaporated off each time in order to remove the ammonia. The syrupy residue was diluted with some water and the solution obtained was clarified with norit. A large volume of absolute alcohol was added to the filtrate. Crystallization of the tripeptide set in after prolonged stirring of the solution. The mixture was left to stand for 2 days in the ice box. The crystals were filtered off and thoroughly washed with hot absolute alcohol. They were dried in a vacuum desiccator over phosphorus pentoxide. Yield, 55 gm. (85.6 per cent of the theory). This material contained traces of chlorine and ammonia. For analysis and rotation it was recrystallized twice from water and alcohol. In each case the substance was dissolved in a small amount of water. The solution was clarified with norit and about 9 volumes of absolute alcohol were added to the filtrate at room temperature. After crystallization had started, the mixture was cooled for 24 hours in ice and water. The crystals were thoroughly washed with hot absolute alcohol; they were then dried in a vacuum oven at 60° and finally in a vacuum desiccator over phosphorus pentoxide. For analysis they were redried at 100°.

---

[5] Abderhalden, E., and Fodor, A., *Ber. chem. Ges.*, **49**, 564 (1916).

No. 496. 5.116 mg. substance: 9.190 mg. CO_2 and 3.570 mg H_2O
0.1000 gm. " : 12.21 cc. 0.1 N HCl (Kjeldahl)
21.700 mg. " : 2.33 cc. N (28.6°, 754 mm.) (Van Slyke)
$C_{10}H_{19}O_4N_3$ (245.17). Calculated. C 48.95, H 7.81, N 17.14, NH_3 5.71
Found. " 48.98, " 7.80, " 17.11, " 5.79
Moisture, none

It had the following rotation.

$$[\alpha]_D^{20} = \frac{-9.69° \times 100}{4 \times 4.9033} = -49.4° \text{ (in water)}$$

0.7355 gm., dry, in 15.00 cc. of solution (0.2 M)

Abderhalden and Fodor[6] found for a levo-leucyl-glycyl-glycine, twice recrystallized from water and alcohol, $[\alpha]_D^{20} = +43.42°$ in water, and for a fraction of tripeptide isolated from the second mother liquor of crystallization $[\alpha]_D^{20} = +45.90°$ in water. A sample from another of their operations showed a rotation of $[\alpha]_D^{20} = +44.79°$ in water.

---

[6] Abderhalden, E., and Fodor, A., *Ber. chem. Ges.*, 49, 565 (1916).

Levo-α-Bromoisocapronyl-Dextro-Leucyl-Glycyl-Glycine

A solution of 88.3 gm. (0.36 mol) of dry dextro-leucyl-glycyl-glycine in 360 cc. of 1.0 N sodium hydroxide (0.36 mol) was cooled in an ice and water mixture during treatment with 77 gm. (0.36 mol) of levo-α-bromoisocapronyl chloride and 360 cc. of 1.0 N sodium hydroxide (0.36 mol), these reagents being added in equivalent proportions with continuous shaking. 10 cc. of ether and then 100 cc. of 5.0 N hydrochloric acid (0.5 mol) were added to the solution. The bromo compound precipitated as a pasty mass. The mixture was then shaken at room temperature until crystallization took place. The material was filtered off and washed with ice water and with petrolic ether. It was then dissolved in absolute alcohol and reprecipitated with a large amount of water. The material obtained (no yield is given, an undetermined amount having been accidentally lost) melted at 186–187° (uncorrected) with decomposition. Its rotation was $[\alpha]_D^{25} = -4.97°$ (in absolute alcohol). On recrystallization from a mixture of absolute ethyl acetate and alcohol (the procedure given below was followed in its essentials) a material of higher rotation, yet still melting at 186–187° (uncorrected), was obtained.

$$[\alpha]_D^{25} = \frac{-0.96° \times 100}{4 \times 4.2220} = -5.68° \text{ (in absolute alcohol)}$$

0.6333 gm. in 15.00 cc. of solution (0.1 M)

This material was recrystallized once more from the same mixture of solvents. There was no increase in rotation. It melted at 188° (uncorrected) or at 190° (corrected), with alteration. The fused substance then decomposed rapidly.

No. 494. 5.405 mg. substance: 9.028 mg. CO_2 and 3.100 mg. H_2O
0.1000 gm. " : 7.16 cc. 0.1 N HCl (Kjeldahl)
0.0969 " " : 0.0433 gm. AgBr (Carius)
$C_{16}H_{28}O_5N_3Br$ (422.16). Calculated. C 45.48, H 6.69, N 9.95, Br 18.93
Found. " 45.54, " 6.41, " 10.02, " 19.01

The substance used for amination was highly purified. It was obtained from 73.5 gm. of original material of rotation $[\alpha]_D^{25} = -4.97°$, which was recrystallized four times in the following way:

Absolute alcohol was added at boiling temperature to suspensions of 1 part of bromo compound in 2 parts by weight of absolute ethyl acetate until the materials were completely dissolved. When necessary, norit was added and the solutions were filtered while hot. To these, 5.3 parts by weight (in respect to the bromo compound) of absolute ethyl acetate were then added. For the first and second crystallizations the solutions were simply cooled to room temperature, but subsequently the mixtures were cooled in ice and water. The crystals were washed with an ethyl acetate-alcohol mixture (1:1, by volume) and finally with heptane. They were dried in a vacuum oven at 60° and then in a vacuum desiccator over phosphorus pentoxide and sodium hydroxide. The yields were as follows: 73.5 gm. → 56.0 gm. → 32.1 gm.; 31.0 gm. → 24.6 gm. → 19.7 gm. The rotations of the materials obtained after the second and fourth recrystallizations were identical; namely, $[\alpha]_D^{30} = -5.64°$, in absolute alcohol ($\alpha_D^{30} = -0.95°$, 4 dm. tube; 0.6333 gm. in 15.03 cc. of solution).

Dextro-Leucyl-Dextro-Leucyl-Glycyl-Glycine

A solution of 18.7 gm. of pure levo-α-bromoisocapronyl-dextro-leucyl-glycyl-glycine in 187 gm. of ammonium hydroxide of sp. gr. 0.90 was kept at room temperature for 4½ days. It was then diluted with water, treated with norit, and concentrated to dryness under reduced pressure. The material was suspended in a small amount of water and absolute alcohol was added to the volume of 1500 cc. This mixture was cooled for 12 hours in the ice box. The solid was filtered off, thoroughly washed with hot absolute alcohol, and while still moist placed in a vacuum desiccator over phosphorus pentoxide and kept there until completely dry. Yield, 11.5 gm. (72 per cent of the theory). This material was again purified in the way indicated above, with the cooling below room temperature omitted. It was then dried in a vacuum oven at 60° and finally over phosphorus pentoxide.

No. 497. 4.965 mg. substance: 9.710 mg. CO_2 and 3.700 mg. H_2O
5.374 " " : 0.755 cc. N (31°, 753.7 mm.)
$C_{16}H_{30}O_5N_4$ (358.27). Calculated. C 53.59, H 8.44, N 15.64
Found. " 53.33, " 8.34, " 15.65
Moisture, none

It had the following rotation.

$$[\alpha]_D^{30} = \frac{-1.14° \times 100}{4 \times 3.568} = -8.0° \pm 0.2° \text{ (in pyridine-water)}$$

0.5374 gm. + 5.50 cc. of pyridine + water to volume 15.06 cc. (0.1 M solution)

Chloroacetyl-Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine

A solution of 8.4 gm. (0.0235 mol) of dry dextro-leucyl-dextro-leucyl-dextro-leucine (9.1 gm. of tripeptide containing 7.74 per cent of moisture)[7] in 23.5 cc. of 1.0 N sodium hydroxide (0.0235 mol) was cooled in an ice and water mixture during treatment with 8.0 gm. (0.0705 mol) of chloroacetyl chloride and 77.0 cc. of 1.0 N sodium hydroxide (0.0770 mol), these reagents being added in equivalent proportions with continuous shaking. 20 cc. of 5.0 N hydrochloric acid (0.1000 mol) were added and a crystalline precipitate of the chloro compound was obtained at once. The crystals were filtered off and washed with ice water and then with petrolic ether; they were again washed with water and with petrolic ether. This material was dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. It weighed 10.2 gm. (theory, 10.2 gm.). The solution of this material in hot absolute alcohol was clarified with norit and filtered. Water was gradually added to the solution until crystallization set in. The mixture was left to cool slowly to room temperature and then a further amount of water was added. The crystals deposited were filtered off, washed with dilute alcohol, and then with petrolic ether. 8.0 gm. of dry substance were recovered. It melted at 198–199° (uncorrected), with no apparent alteration. After some time, however, the fused material suffered decomposition.

No. 492. 4.995 mg. substance: 10.100 mg. CO_2 and 3.610 mg. H_2O
5.800 " " : 0.484 cc. N (24°, 758.8 mm.)
15.430 " " : 5.300 mg. AgCl (Carius)
$C_{20}H_{36}O_5N_3Cl$ (433.78). Calculated. C 55.33, H 8.37, N 9.69, Cl 8.17
Found. " 55.14, " 8.08, " 9.58, " 8.49

---

[7] Levene, P. A., Steiger, R. E., and Marker, R. E., *J. Biol. Chem.*, **93**, 615 (1931).

It had the following rotation.

$$[\alpha]_D^{27} = \frac{+2.98° \times 100}{4 \times 1.0465} = +71.2° \text{ (in absolute alcohol)}$$

0.1575 gm. in 15.05 cc. of solution

Abderhalden and Fleischmann[8] found for chloroacetyl-levo-leucyl-levo-leucyl-levo-leucine $\alpha_D^{20} = -0.8°$ (1 dm. tube) or $[\alpha]_D^{20} = -76.19°$, employing a solution of 0.105 gm. in alcohol, the volume of the solution being 10 cc. Their material melted at 193° with decomposition.

Glycyl-Dextro-Leucyl-Dextro-Leucyl-Dextro-Leucine

A mixture of 7.0 gm. of purified chloroacetyl-dextro-leucyl-dextro-leucyl-dextro-leucine with 10 times this weight of ammonium hydroxide of sp. gr. 0.90 was kept at room temperature for 3 days (temperature about 25°) during which time it was occasionally shaken. A cloudy solution was finally obtained; it was diluted with water and clarified with norit. The filtrate was concentrated under reduced pressure. The ammonia thus being removed, the tetrapeptide separated in the form of a voluminous gel. This was filtered off and thoroughly washed with absolute alcohol. The filtrate was concentrated under reduced pressure. It then yielded a second fraction of polypeptide. The two fractions were combined. This material weighed 4.7 gm. It was suspended in absolute alcohol and small amounts of ammonium hydroxide, of sp. gr. 0.90, were added gradually until an almost clear solution was obtained. This was clarified with norit. The filtrate was concentrated under reduced pressure for the removal of the ammonia and the recovery of the tetrapeptide. Alcohol was freely run into the suspension obtained while the concentration was continued. The substance was filtered off, thoroughly washed with absolute alcohol, and was then dried in a vacuum desiccator over phosphorus pentoxide.

No. 495. 5.251 mg. substance: 11.075 mg. CO_2 and 4.270 mg. H_2O
4.030 " " : 0.477 cc. N (29°, 755.4 mm.)
$C_{20}H_{38}O_5N_4$ (414.33). Calculated. C 57.92, H 9.24, N 13.52
Found. " 57.51, " 9.09, " 13.29
Moisture, 0.73 per cent

---

[8] Abderhalden, E., and Fleischmann, R., *Fermentforschung*, 9, 529 (1926–28).

It had the following rotation.

$$[\alpha]^{30}_{\mathrm{D}} = \frac{+6.7° \times 100}{4 \times 2.035} = +82.3° \pm 0.6° \text{ (in 1.0 N NaOH)}$$

0.3053 gm., dry, in 15.0 cc. of solution

TABLE II

Action of Sodium Hydroxide at 25° on Tetrapeptides

Compound	Dextro-leucyl-dextro-leucyl-glycyl-glycine	Glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine
Experiment No.	1, 2, 3	4 and 5
Amount of substance used, *gm.*	0.8957 (1) 0.8957 (2) 0.8957 (3) (0.0025 mol)	0.8287 (4) 0.8287 (5) (0.0020 mol)
1.0 N NaOH used, *cc.*	25.0 (1, 2, 3)	20.0 (4, 5)
Action of alkali at 25°, *days*	8 (1, 2, 3)	8 (4, 5)
Total N in hydrolysate with 20 cc. 20 per cent HCl (36 hrs. at 100°), *gm. per 100 cc.*	0.6523 (1) 0.6512 (2) 0.6557 (3)	0.5209 (4) 0.3438* (5)
$\frac{\text{Amino N}}{\text{Total N}}$, *per cent*	99.8 (1) 99.2 (2) 98.3 (3)	98.3 (?) (4) 99.0 (5)
$\alpha_{1}{}^{30}_{\mathrm{D}}$ (4 dm. tube), *degrees*	-1.61 (1) -1.58_5 (2) -1.58_5 (3)	-1.82 (4) -1.19 (5)
$[\alpha_1]^{30}_{\mathrm{D}}$ of leucine in solution, *degrees*	-13.1_9 (1) -13.0_0 (2) -12.9_1 (3)	-12.4_4 (4) -12.3_3 (5)

* The hydrolysate was diluted with 20 per cent HCl before examination.

The reading (correct to ±0.05°) was made as soon as the field appeared clear. The rotation of this solution (at about 31°) dropped by 25 per cent in the course of 48 hours because of hydrolysis and racemization. This perhaps explains why Abderhalden and Fleischmann[8] found for glycyl-levo-leucyl-levo-leucyl-levo-leucine $\alpha^{20}_{\mathrm{D}} = -1.6°$ (1 dm. tube) or $[\alpha]^{20}_{\mathrm{D}} = -78.63°$, employing a solution of 0.2035 gm. of substance in 1.0 N sodium hydroxide, the volume of the solution being 10 cc.

2. Action of Sodium Hydroxide on Tetrapeptides (Racemization)

All the data are recorded in Table II. The alkaline solutions of the tetrapeptides prepared were kept for 8 days in a thermostat at 25°. The solutions were then acidified with hydrochloric acid and concentrated to dryness under reduced pressure in the way indicated in a previous paper.[9] The dry residues were then taken up in 20 cc. of 20 per cent hydrochloric acid. The test-tubes were sealed and immersed for 36 hours in a steam-heated water bath at 100°. The suspensions obtained were filtered in order to remove the sodium chloride that had separated at room temperature. The total nitrogen was determined on 5 cc. and the amino nitrogen

TABLE III

Hydrolysis of Tetrapeptides by Hydrochloric Acid at 100°

Compound	Dextro-leucyl-dextro-leucyl-glycyl-glycine	Glycyl-dextro-leucyl-dextro-leucyl-dextro-leucine
Experiment No.	6 and 7	8 and 9
Amount of substance used, *gm.*	0.8957 (6) 0.8957 (7) (0.0025 mol)	0.8287 (8) 0.8287 (9) (0.0020 mol)
Amount of NaCl added, *gm.*	0.2923	0.2923
Total N in hydrolysate with 20 cc. 20 per cent HCl (36 hrs. at 100°), *gm. per 100 cc.*	0.6683 (6) 0.6730 (7)	0.5215 (8) 0.5203 (9)
$\frac{\text{Amino N}}{\text{Total N}}$, *per cent*	100.0 (6) 98.5 (7)	100 (8) — (9)
$\alpha_{0\mathrm{D}}^{30}$ (4 dm. tube), *degrees*	−2.03 (6) −2.02 (7)	−2.38 (8) -2.35_5 (9)
$[\alpha_0]_{\mathrm{D}}^{30}$ of leucine in solution, *degrees*	-16.2_3 (6) -16.0_1 (7)	-16.2_6 (8) -16.1_2 (9)

---

[9] Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, **86**, 715 (1930).

on 1 cc. of solution. The loss of optical activity is given in per cent by the ratio

$$\frac{[\alpha_0]_D^t - [\alpha_1]_D^t}{[\alpha_0]_D} \times 100$$

The values $[\alpha_0]_D^t$ of the blanks are given in Table III.

3. Action of Hydrochloric Acid on Tetrapeptides (Hydrolysis)

The tetrapeptides were hydrolyzed with 20 per cent hydrochloric acid in the presence of sodium chloride in sealed tubes immersed for 36 hours in a steam-heated water bath at 100°. The total nitrogen was determined on 5 cc. and the amino nitrogen on 1 cc. of solution. All the data are recorded in Table III. The values $[\alpha_0]_D^t$ were thus obtained.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, September, 1932,
Vol. 97, No. 3, pp. 751-761]

ON THE STRUCTURE OF THE SO CALLED 5-METHYL GLUCOSE OF OHLE AND VON VARGHA

BY P. A. LEVENE AND ALBERT L. RAYMOND

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 21, 1932)

In continuation of the work on the nature of the glycoside formation in the partially substituted sugars,[1] a study of the 5-methyl glucose described by Ohle and von Vargha[2] was undertaken by us. On the basis of previous experience, a substance of this structure was expected to form but one glucoside, the furanoside, and it was therefore surprising to find indication of the formation of two glucosides. These were formed, moreover, at approximately the same rates as in the case of unsubstituted glucose. The formation of two glucosides from 5-methyl glucose would require either the existence of glucosides with ring structures other than $<1, 4>$ and $<1, 5>$, or else the migration of the methyl group in the course of glucoside formation. Further study, however, has convinced us that no irregularity had occurred but that instead an incorrect structure has been assigned by Ohle to his substance, which in reality is 6-methyl glucose.

The following observations led to this conclusion. The osazone of the substance described by Ohle has a melting point (180–185°) which is very close to that last reported for 6-methyl glucosazone (184–187° corrected).[3] The rotation of the osazone of the "5-methyl" glucose was reported as $-101.9°$ in pyridine,[2] and this we have confirmed. However, in alcohol the "5-methyl" glucosazone has initial and final rotations of $-70°$ and $-44°$ respectively. These values are practically identical with those reported by

---

[1] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **96**, 449 (1932).

[2] Ohle, H., and von Vargha, L., *Ber. chem. Ges.*, **62**, 2435 (1929).

[3] Helferich, B., and Günther, E., *Ber. chem. Ges.*, **64**, 1276 (1931).

Helferich and Günther[3] and by Ohle and von Vargha[4] for 6-methyl glucosazone; namely, initial −70.3° and −69.6°, and final, −46.9° and −46.4°.

Methylation of the methylglucoside resulting after long heating of a solution of Ohle's "5-methyl" glucose in methyl alcohol containing hydrogen chloride, gave a tetramethyl methylglucoside which was hydrolyzed to a tetramethyl glucose. This was crystallized and found to have the properties of 2,3,4,6-tetramethyl glucose. The tetramethyl glucose was further identified by conversion to the anilide, identical with that from 2,3,4,6-tetramethyl glucose. From 5-methyl glucose this substance could have been formed only by actual migration of the methyl group from position (5), an improbable assumption. Moreover, this possibility is contradicted by the curves of lactone formation of the "5-methyl" gluconic acid which indicate two lactones, formed at rates which are close to those found for the corresponding lactones of unsubstituted glucose. As the lactone formation was measured at room temperature and in only slightly acid solution, migration of a methyl group would appear most unlikely.

For further evidence, the monomethyl glucose was converted to the tetracetate, and through the acetobromo derivative, to the triacetyl methyl methylglucoside. Both of these derivatives were found to have the properties reported by Helferich and Günther for the corresponding derivatives of 6-methyl glucose.

All of these results lead to the conclusion that the crystalline "5-methyl" glucose described by Ohle and von Vargha is the 6-methyl glucose, previously obtained by Helferich and Becker,[5] though in amorphous state. As a consequence of this conclusion, the structure of the "trimethyl glucose" of Pacsu,[6] shown by Shinle[7] to be a monomethyl glucose, was in need of reinvestigation inasmuch as the 5-methyl structure was excluded by Schinle on the basis of the claims of Ohle. However, it will be shown in a subsequent publication that the assumption of Shinle is probably correct.

Work on the synthesis of 5-methyl glucose is now in progress.

---

[4] Ohle, H., and von Vargha, L., *Ber. chem. Ges.*, **62**, 2425 (1929).

[5] Helferich, B., and Becker, J., *Ann. Chem.*, **440**, 1 (1924).

[6] Pacsu, E., *Ber. chem. Ges.*, **57**, 849 (1924); **58**, 1455 (1925).

[7] Schinle, R., *Ber. chem. Ges.*, **65**, 315 (1932).

EXPERIMENTAL

Methyl Monoacetone Glucose—6-Tolylsulfo monoacetone glucose was prepared from monoacetone glucose following the procedure of Ohle and von Vargha.[2] In general the yields were not quite as high as those reported by them.

The 6-tolylsulfo monoacetone glucose was converted to the methyl monoacetone glucose by a slight modification of Ohle's[2] procedure, the isolation of the anhydromonoacetone glucose being omitted. 50 gm. of tolylsulfo monoacetone glucose were dissolved in 100 cc. of methyl alcohol, cooled in an ice-salt mixture, and added with vigorous shaking to a solution of 3.75 gm. of metallic sodium in 100 cc. of methyl alcohol similarly cooled in an ice-salt mixture. The product was allowed to stand 20 minutes in the freezing mixture and an ice-cold solution of 7.5 gm. of metallic sodium in 125 cc. of methyl alcohol was then added with shaking. A separation of sodium toluenesulfonate occurred immediately. The mixture was allowed to stand at room temperature with occasional shaking for 2 days and was then cooled in ice water. Hydrochloric acid was added until the solution became just acid to phenolphthalein (pH about 7.6). 2 volumes of ether were added and the precipitate was filtered off and washed with more ether. The filtrate and washings were combined and concentrated under reduced pressure to a syrup which was taken up in chloroform. The chloroform solution was washed twice with water, dried with sodium sulfate, and concentrated under reduced pressure to a syrup. This was taken up in ether and the solution was cooled in the refrigerator. Pentane was added just to turbidity and the mixture was seeded. The seed crystals were obtained by distilling a portion of the material under greatly diminished pressure as described by Ohle. The yield of crude crystalline product was 19 gm. and after one recrystallization from ether-pentane, 15 gm.

Several lots of the methyl monoacetone glucose were combined and recrystallized as above.

The substance had the composition of a methyl monoacetone hexose.

4.595 mg. substance: 8.635 mg. CO_2 and 3.225 mg. H_2O
3.395 " " : 3.475 " AgI
$C_{10}H_{18}O_6$. Calculated. C 51.26, H 7.76, OCH_3 13.24
234.1 Found. " 51.24, " 7.85, " 13.51

The product sintered at about 70° and then melted at 71–72°. It had a rotation, in chloroform, of $[\alpha]_D^{20} = \frac{-0.24° \times 100}{2 \times 2.0} = -6.0°$. Ohle and von Vargha[2] give 71–72° as the melting point and $-6.4°$ as the rotation.

Monomethyl Glucose—The methyl monoacetone glucose described above was hydrolyzed exactly as described by Ohle and von Vargha[2] and the product was recrystallized twice from absolute alcohol. The composition corresponded to a monomethyl hexose.

4.725 mg. substance: 7.511 mg. CO_2 and 3.040 mg. H_2O
5.891 " " : 7.435 " AgI
$C_7H_{14}O_6$. Calculated. C 43.28, H 7.27, OCH_3 15.93
194.1 Found. " 43.34, " 7.19, " 16.66

The substance had a melting point which varied greatly with the rate of heating. If kept at 138° for several minutes it melted to a clear liquid, but if heated rapidly, it softened at 150° and melted at 153–154°. Ohle and von Vargha[2] reported 143–144° as the melting point. The optical rotation in water of our product was 101.8° 1 minute after dissolving, 86.0° at 30 minutes, 59.5° at 3 hours, and 56.0° at 5 and at 24 hours. A second sample had an initial rotation of 104.5° and an equilibrium rotation, after 5 hours, of 58.5°. Ohle and von Vargha[2] reported 101.2° 3 minutes after dissolving, and 59.92° at equilibrium after 3 hours.

Methyl Glucosazone—0.5 gm. of the methyl glucose was converted to the osazone exactly as described by Ohle and von Vargha.[2] The product, which formed readily, was recrystallized from alcohol, in which it was fairly soluble.

The analysis corresponded to a methyl hexosazone.

4.880 mg. substance: 0.651 cc. N (28° and 761 mm.)
4.650 " " : 3.070 mg. AgI
$C_{19}H_{24}O_4N_4$. Calculated. N 15.06, OCH_3 8.33
372.2 Found. " 15.14, " 8.72

On heating the recrystallized material, it contracted at 180°, melted at 183°, and decomposed at 192°. Ohle and von Vargha[2] had reported 180° with decomposition as the melting point of their material. Helferich and Günther[3] gave 184–187° (corrected) as the melting point of the pure 6-methyl glucosazone, and Ohle and von Vargha[4] found 178° as the melting point of the osazone of 6-methyl glucose prepared through isodiacetone glucose.

The rotation of the product, in pyridine, was −102° initial, and −28° at equilibrium after 24 hours. Ohle and von Vargha[2] reported −101.9° initial, but were unable to observe the end value due to coloration of the solution. Our own solution darkened only slightly in 6 days. The rotation of our product in absolute alcohol was −70° 5 minutes after dissolving and −44° at equilibrium after 24 hours. Helferich and Günther[3] reported −70.3° initial and −46.9° at equilibrium for the rotations, in alcohol, of the osazone of 6-methyl glucose, and Ohle and von Vargha[4] reported −69.6° initial and −46.4° at equilibrium for the osazone of 6-methyl glucose prepared from methyl isodiacetone glucose.

Tetracetyl-6-Methyl Glucose—3 gm. of the "5-methyl" glucose were acetylated according to the procedure described by Helferich for the 6-methyl glucose. The product crystallized easily and was twice recrystallized from a little absolute alcohol. The composition corresponded to a tetracetyl methyl hexose.

4.695 mg. substance: 8.568 mg. CO_2 and 2.550 mg. H_2O
6.075 " " : 4.001 " AgI

$C_{15}H_{22}O_{10}$.	Calculated.	C 49.70, H 6.12, OCH_3 8.55
362.2	Found.	" 49.76, " 6.07, " 8.69

The melting point was 95–96° and the rotation, in chloroform, was $[\alpha]_D^{20} = \frac{+0.86° \times 100}{2 \times 2.0} = +21.5°$. Helferich reports 91–93° (corrected) as the melting point and +20.9° as the rotation, in chloroform, for β-tetracetyl-6-methyl glucose.

Triacetyl-6-Methyl-β-Methylglucoside—The syrupy tetracetate from 9.5 gm. of the "5-methyl" glucose was treated with 50 cc. of a saturated solution of hydrogen bromide in glacial acetic acid without preliminary attempts at crystallization. After standing for 2 hours at room temperature the mixture was poured onto ice, ice water was added, and the solution was extracted three times with chloroform. The extracts were washed three times with ice water, dried with sodium sulfate, and concentrated under reduced pressure to a thick syrup. This was taken up in 100 cc. of dry methyl alcohol, cooled to 10°, and 10 gm. of dry silver oxide were added. The mixture was shaken vigorously. A test after 10 minutes indicated that practically no bromide remained. The mixture was filtered, then refiltered with charcoal, and concentrated under reduced pressure to a syrup. This was dissolved in ether and cooled. Pentane was added just to turbidity. On standing overnight in the ice box the material crystallized. A second crop was obtained on concentrating the mother liquors. The total yield was 12.4 gm. The material was recrystallized twice from absolute alcohol and once from ether.

The composition corresponded to a triacetyl methyl methylhexoside.

5.001 mg. substance: 9.195 mg. CO_2 and 2.920 mg. H_2O
4.775 " " : 6.685 " AgI
$C_{14}H_{22}O_9$. Calculated. C 50.27, H 6.64, OCH_3 18.55
334.2 Found. " 50.17, " 6.53, " 18.48

The product melted at 104–105° and had a rotation, in chloroform, of $[\alpha]_D^{20} = \frac{-0.58° \times 100}{2 \times 2.0} = -14.5°$. Helferich and Günther[3] give a melting point of 107–108° and a rotation of $-12.4°$ for the triacetyl-β-methylglucoside of the 6-methyl glucose.

Methyl Methylglucoside—5 gm. of the monomethyl glucose were dissolved in 250 cc. of dry methyl alcohol containing 0.5 per cent hydrogen chloride. The solution was sealed in a round bottom flask and heated in a bath of boiling carbon tetrachloride for 43 hours. The specific rotation of the solution, after cooling, was +115°. The hydrogen chloride was removed with silver oxide and the silver precipitate was washed with warm methyl alcohol. The methyl alcohol solution and washings were combined and concentrated under reduced pressure to a syrup. Of this syrup 0.4 gm. were removed for analysis and dried over phosphorus pentoxide under greatly reduced pressure. Although not a pure product, the composition corresponded fairly well to a methyl methylhexoside.

3.452 mg. substance: 5.700 mg. CO_2 and 2.500 mg. H_2O
3.710 " " : 9.170 " AgI
$C_8H_{16}O_6$. Calculated. C 46.15, H 7.76, OCH_3 29.81
208.1 Found. " 45.02, " 8.10, " 32.62

Tetramethyl Methylglucoside—The product prepared above was methylated by the Purdie method with 45 cc. of methyl iodide as the solvent. The solution was initially turbid. The product was isolated in the usual manner by repeated extraction with hot chloroform. The solution was concentrated under reduced pressure to a syrup and this was redissolved in 45 cc. of methyl iodide, giving a clear solution. After remethylation the product was isolated in the same manner. The chloroform extract was concentrated under reduced pressure to a syrup and this was distilled under greatly reduced pressure. Yield 4.2 gm. The product had a rotation, in alcohol, of $[\alpha]_D^{20} = +101°$, and a refractive index of $n_D^{19} = 1.4413$. The composition corresponded to a tetramethyl methylhexoside.

5.980 mg. substance: 11.455 mg. CO_2 and 4.685 mg. H_2O
4.670 " " : 21.311 " AgI
$C_{11}H_{22}O_6$. Calculated. C 52.76, H 8.86, OCH_3 61.95
250.2 Found. " 52.24, " 8.76, " 60.23

Tetramethyl Glucose—3.8 gm. of the above product were dissolved in 75 cc. of 1.5 N hydrochloric acid and heated on the steam bath for 7 hours. The hydrochloric acid was removed with silver oxide and the silver salts were extracted once with warm water and twice with boiling acetone. The combined extracts were concentrated under reduced pressure to a syrup. On seeding with a crystal of 2,3,4,6-tetramethyl glucose, the syrup crystallized immediately. The product was twice recrystallized from pentane containing 0.5 per cent of ether. It melted at 88–90° and had a specific rotation, in alcohol, of 102° 2 minutes after dissolving, and 83.5° at equilibrium after 48 hours. The melting points recorded in the literature range from 88–104°. The rotation in alcohol is reported as 104.9° initial, and 83.9° at equilibrium.

Tetramethyl Glucose Anilide—0.5 gm. of the above tetramethyl glucose was dissolved in 2 cc. of absolute alcohol, 0.58 cc. of freshly distilled aniline (3 mols) were added, and the mixture was refluxed on the steam bath for 3 hours. On cooling, the product crystallized. It was filtered off, washed with a little absolute alcohol, and recrystallized from absolute alcohol. It melted at 137–138° and had a rotation, in methyl alcohol containing 0.001 per cent hydrochloric acid, of +64.0° at equilibrium. Greene and Lewis[8] report a melting point of 138° and a final rotation of +59° in methyl alcohol containing 0.001 per cent hydrochloric acid.

---

[8] Greene, R. D., with Lewis, W. L., *J. Am. Chem. Soc.*, 50, 2813 (1928).

Methyl Gluconic Acid and Lactone Formation—To 5 gm. of the methyl glucose in 90 cc. of water, was added a solution of 12.5 gm. of iodine and 25 gm. of barium iodide in 60 cc. of water. The mixture was stirred mechanically and 300 cc. of 0.5 N barium hydroxide solution were dropped in, 20 minutes being required for the addition. The mixture was stirred for an additional 15 minutes and then acidified with sulfuric acid. Sulfur dioxide was passed in until the iodine was exactly reduced, and the halide was removed by adding moist silver carbonate. Acid was added as required to keep the mixture acid to Congo paper. The silver precipitate was filtered off, resuspended in dilute sulfuric acid, refiltered, and washed with dilute sulfuric acid. The combined filtrates were saturated with hydrogen sulfide, and aerated to remove the excess. A warm saturated solution of barium hydroxide was added to a pH of 8.5 and the mixture of silver sulfide and barium sulfate was filtered off and washed with water. The combined filtrate and wash water were concentrated under reduced pressure to about 100 cc., readjusted to pH 8.5, and filtered with charcoal. This filtrate was concentrated to a thick syrup and then dried over phosphorus pentoxide to a glassy solid. This was powdered and redried to constant weight. Yield 6.7 gm. The composition corresponded to a barium salt of a methyl hexonic acid.

TABLE I

Observed Rotations during Lactone Formation

Time	α	Time	α	Time	α
min.	*degrees*	*hrs.*	*degrees*	*hrs.*	*degrees*
1½	0.00	1	0.54	72	1.10
3	0.09	1½	0.53	144	1.27
8	0.18	3	0.57	288	1.24
15	0.35	6	0.61		
32	0.45	23	0.85		

8.530 mg. substance: 7.315 mg. AgI
12.090 " " : 5.490 " $BaSO_4$
$(C_7H_{13}O_7)_2Ba$. Calculated. OCH_3 11.16, Ba 24.74
555.6 Found. " 11.31, " 26.72

The rotation, in water, was $[\alpha]_{\text{D}}^{21} = \frac{+1.06° \times 100}{2 \times 2.0} = +26.5°$.

0.250 gm. of this product was dissolved in water, 0.97 cc. (2 mols per mol of Ba) of 1.0 N hydrochloric acid was added, the mixture was diluted immediately to a volume of 5.0 cc., and the rotation was measured in a 2 dm. tube with sodium (D) light. The observed rotations are given in Table I and the specific rotations (calculated on the basis of barium salt) are plotted in Fig. 1.

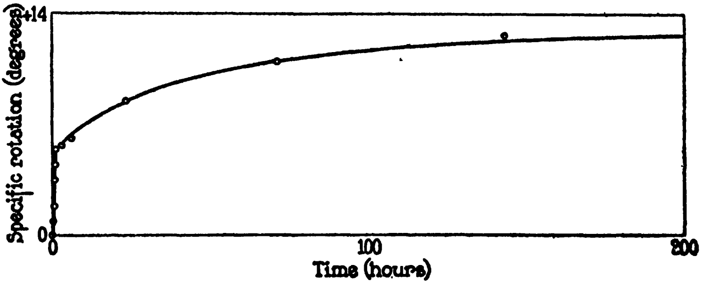


FIG. 1. Optical rotation during lactone formation at 25°

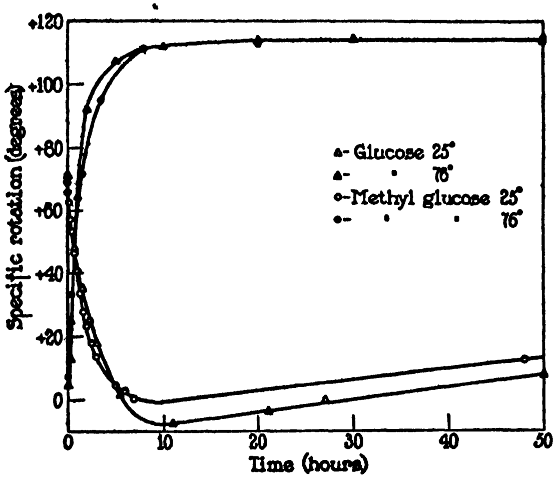


FIG. 2. Optical rotation during glucoside formation at 25° and at 76°. Glucose and methyl glucose.

Glucoside Formation—The methods and technique have been previously described[9] and need not be repeated in detail here. A 0.344 molal solution of the sugar in dry methyl alcohol containing 0.5 per cent of dry hydrogen chloride was used. Experiments were made at room temperature, and in sealed tubes heated in a bath of boiling carbon tetrachloride. The rotations were measured at 20° with sodium (D) light. 4 dm. tubes were used for the room temperature experiments and 2 dm. for the others. The specific rotations calculated from the data are plotted in in Fig. 2. The corresponding curves for glucose are reproduced from an earlier paper for purposes of comparison.

TABLE II

Glucoside Formation at 25° from Reduction Determinations

Time	Cc. 0.01 N thiosulfate		Mg.		Free sugar, per cent		Distribution of sugar, per cent		
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis	Free	Glucoside	
								γ	Normal
hrs.									
0	10.88	7.03	10.55	6.82*	106	102	106	−4	−2
1	7.86	6.93	7.62	6.73*	76	101	76	25	−1
3	4.32	6.90	4.19	6.70*	42	100	42	58	0
7	2.80	6.74	2.72	6.54*	27	98	27	72	2
24	2.39	8.82	2.32	8.55	23	86	23	63	14
48	2.47	7.64	2.40	7.41	24	74	24	50	26

* The total sugar present in these samples was 6.67 mg. In all the others it was 10.0 mg.

In order to determine analytically the character of the glucosides which were formed, the method previously developed[1,9] was used. This consisted in determining the amount of reducing sugar present in a particular sample, before and after hydrolysis with dilute acid. The difference is assumed to represent the amount of γ-glucoside present in the sample. The limitations of the method, as well as the necessary corrections, have been previously discussed in detail.

The micro Willstätter[1] method was used without modification. This was found to give practically theoretical values for the methyl glucose both before and after heating with acid, so that no correction was required. A sample of the glucoside which had been formed by heating the acid methyl alcohol solution at 76° for 8 hours was hydrolyzed with 0.1 N hydrochloric acid at 100° for 10 minutes and then analyzed for reducing sugar. The amount present was, within the limits of experimental error, no more than before the hydrolysis so that this correction was likewise omitted.

---

[9] Levene, P. A., Raymond, A. L., and Dillon, R. T., *J. Biol. Chem.*, **95**, 699 (1932).

The data, and the amounts of γ-glucoside, normal glucoside, and free sugar, calculated from them are given in Table II. Comparison with the results on glucose in the earlier papers[1,9] shows that the rates of formation of the γ-glucosides are closely alike in the two sugars, as are those for the normal glucosides.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, September, 1932, Vol. 97, No. 3, pp. 763–775]

THE SUBSTITUTION OF GLUCOSE IN POSITION (4). I

BY P. A. LEVENE AND ALBERT L. RAYMOND

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 21, 1932)

For some time we have been desirous of having intermediates for the synthesis of glucose derivatives substituted in position (4), in particular for the synthesis of 4-methyl glucose, 4-phospho glucose, and of certain disaccharides. Schinle[1] has recently ascribed the structure of 4-methyl glucose to the "trimethyl" glucose of Pacsu,[2] but the structure has not been well established, and the method of preparation is such as to be of little value for the synthesis of derivatives other than the methyl glucose. Helferich[3,4,5] has also described two possible intermediates, 1,2,3,6-tetracetyl glucose and 2,3,6-triacetyl-β-methylglucoside, but their preparation (by isomerization of the corresponding 2,3,4 derivatives) is too complicated.

The procedures we have been engaged in developing are similar in certain respects to those published by Oldham and Rutherford[6] but avoid the tedious introduction and removal of the tolylsulfo groups. Benzylidene-β-methylglucoside has been shown to be 4,6-benzylidene,[7] and as the ring is in position (5), positions (2) and (3) are unoccupied. By benzoylation of this derivative and subsequent removal of the benzylidene group by careful hydrolysis,

---

[1] Schinle, R., *Ber. chem. Ges.*, **65**, 315 (1932).

[2] Pacsu, E., *Ber. chem. Ges.*, **57**, 849 (1924); **58**, 1455 (1925).

[3] Helferich, B., and Klein, W., *Ann. Chem.*, **450**, 219 (1926); **455**, 173 (1927).

[4] Helferich, B., Bredereck, H., and Schneidmüller, A., *Ann. Chem.*, **458**, 111 (1927).

[5] Helferich, B., and Bredereck, H., *Ber. chem. Ges.*, **64**, 2411 (1931).

[6] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

[7] Ohle, H., and Spencker, K., *Ber. chem. Ges.*, **61**, 2387 (1928).

2,3-dibenzoyl-β-methylglucoside was obtained. This substance, on partial benzoylation, gave, in good yield, a crystalline tribenzoate which appears to be 2,3,6-tribenzoyl-β-methylglucoside. This conclusion rests largely upon the fact that the acetyl derivative of this tribenzoate is not identical with the known 6-acetyl-2,3,4-tribenzoyl-β-methylglucoside already known.

The tribenzoate, upon methylation, yielded a product which has not yet been brought to crystallization. Debenzoylation with barium oxide in methyl alcohol, and reacetylation, gave a crystalline product with the composition of a methyl triacetyl methylglucoside. If the benzoate is really 2,3,6-tribenzoyl-β-methylglucoside, then this product should be triacetyl-4-methyl-β-methylglucoside. Schinle, as mentioned above, considers Pacsu's "trimethyl" glucose to be 4-methyl glucose and we have therefore prepared some of this substance and have converted it, through the acetobromo compound, into the methyl triacetyl-β-methylglucoside. The product is identical with the derivative described above.

Inasmuch as in the case of Schinle's substance the choice of the structure lies between that of 4-methyl and 5-methyl glucose,[8] and as our substance, being derived from a normal glucoside, cannot be substituted in position (5), it seems justifiable to conclude that both products, the one by Schinle and the one by us, have the structure of 4-methyl-β-methylglucoside.

Although the tribenzoate described above is useful for methylation, the relative stability of the benzoyl groups renders the substance unsuitable for the preparation of certain derivatives, such as the phosphoric ester. For this purpose advantage may be taken of the corresponding acetyl compounds. 2,3-Diacetyl-β methylglucoside was prepared by careful hydrolysis of the 2,3-diacetyl-4,6-benzylidene-β-methylglucoside, and was then partially acetylated, giving in good yield a crystalline triacetyl-β-methylglucoside. This was found to have the same melting point as the 2,3,6-triacetyl-β-methylglucoside of Helferich, mentioned above, but the rotation was somewhat different (−59.0° as against the value −64.9° reported by Helferich[4]). The *p*-tolylsulfo derivative of our triacetyl compound had a rotation and melting point which agreed quite well with those reported by Helferich for the corresponding *p*-tolylsulfo derivative of his triacetyl compound, and not at all with those for the triacetyl-6-*p*-tolylsulfo-β-methylglucoside. It thus seems probable that our triacetyl derivative is identical with that of Helferich.

---

[8] Schinle assigned this structure since the methyl glucose was not identical with the already described 2-, 3-, 5-, and 6-methyl glucoses. We have since shown that the so called 5-methyl glucose of Ohle and von Vargha is really 6-methyl glucose (Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **97**, 751 (1932)) so that the 5-methyl structure may again be regarded as a possibility for the monomethyl glucose from the mercapto derivative.

We may mention that in addition to the acetyl derivative of the tribenzoate, the *p*-tolylsulfo tribenzoyl-β-methylglucoside was prepared, but attempts at debenzoylation led to the extensive removal of the tolylsulfo group so that this substance was not further utilized.

The dibenzoyl glucoside, prepared as an intermediate, has not yet been crystallized. Josephson,[9] on the other hand, prepared a substance to which he tentatively ascribed the structure of 2,3-dibenzoyl-β-methylglucoside, and which he obtained in crystalline form. He also described the diacetate, 2,3-dibenzoyl-4,6-diacetyl-β-methylglucoside, melting point 166°. The diacetate of our syrupy dibenzoyl compound, obtained crystalline and in excellent yield, melts at 131–132°, so that we are inclined to question the structures assigned by Josephson to his compounds.

We thus appear to have convenient methods for the preparation of 2,3,6-triacetyl and tribenzoyl-β-methylglucosides. We intend, first, to obtain further confirmation of their structure, and secondly, to make use of them for the synthesis of 4-phospho glucose, of 4-methyl glucose, and of disaccharides.

EXPERIMENTAL

2,3-Dibenzoyl-β-Methylglucoside—β-Methylglucoside was converted to the benzylidene derivative, and benzoylated as described in the literature.[7] The 2,3-dibenzoyl-β-methylglucoside was recrystallized and then hydrolyzed. 10 gm. were dissolved in 90 cc. of acetone and 10 cc. of 1 N hydrochloric acid were added. The mixture was refluxed on the steam bath for 5 hours (the optical rotation was constant after 4 hours) and then cooled. The acid was removed by shaking with silver oxide, and the filtrate from this was refiltered with charcoal. After concentrating under reduced pressure until two layers formed, the mixture was three times extracted with pentane, which was decanted off. The remaining product was taken up in chloroform and washed with sodium bisulfite solution to remove the last traces of benzaldehyde. After washing twice with water, the chloroform solution was dried with sodium sulfate and concentrated to a syrup. A portion of this was dried over phosphorus pentoxide under greatly reduced pressure, powdered, and redried. Although not a crystalline product, the analysis corresponded fairly well to a dibenzoyl methylhexoside.

---

[9] Josephson, K., *Ber. chem. Ges.*, **62**, 317 (1929).

4.856 mg. substance: 11.075 mg. CO_2 and 2.390 mg. H_2O
5.500 " " : 3.530 " AgI
$C_{21}H_{22}O_8$. Calculated. C 62.66, H 5.52, OCH_3 7.71
402.2 Found. " 62.20, " 5.50, " 8.47

As yet this substance has not been crystallized. Its rotation, in chloroform, was $[\alpha]_D^{20} = \frac{+3.71° \times 100}{2 \times 2.0} = +92.8°$. Josephson[9] did not report the rotation of his supposed 2,3-dibenzoyl-β-methylglucoside.

2,3-Dibenzoyl-4,6-Diacetyl-β-Methylglucoside—The syrupy dibenzoate from the hydrolysis of approximately 5.5 gm. of dibenzoyl-benzylidene-β-methylglucoside, was dissolved in 50 cc. of dry pyridine, and 4 cc. of acetic anhydride were added. The mixture warmed somewhat, and without cooling, was left for 18 hours at room temperature. 5 cc. of water were added, and after standing for ½ hour, ice water was added and the mixture was extracted three times with chloroform. The combined extracts were washed once with water, then with dilute, ice-cold sulfuric acid until acid to Congo paper, and then twice more with water. After drying with sodium sulfate, the chloroform extract was concentrated under reduced pressure to a thick syrup. The syrup was dissolved in a little warm ether, and on cooling this extract the product crystallized immediately. Pentane was added and the mixture was allowed to stand in the ice box. The crystals were filtered off and the solution was concentrated and treated in the same manner to secure a second crop. The combined product weighed 5.1 gm. It was three times recrystallized from methyl alcohol. Its composition corresponded to a diacetyl dibenzoyl methylhexoside.

4.170 mg. substance: 9.415 mg. CO_2 and 2.070 mg. H_2O
5.875 " " : 2.831 " AgI
$C_{26}H_{26}O_{10}$. Calculated. C 61.70, H 5.39, OCH_3 6.38
486.2 Found. " 61.56, " 5.55, " 6.36

The substance, on heating, sintered at 123° and melted at 131–132°. Its rotation, in chloroform, was $[\alpha]_D^{20} = \frac{+3.19° \times 100}{2 \times 2.0} = +79.8°$. Josephson[9] reported 166° as the melting point of the product which he suggested to be 2,3-dibenzoyl-4,6-diacetyl-β-methylglucoside.

2,3,6-Tribenzoyl-β-Methylglucoside—The syrupy product from the hydrolysis of 10 gm. of the dibenzoyl-benzylidene-β-methylglucoside was dissolved in 60 cc. of dry pyridine and the solution was stirred mechanically and cooled in running water. A solution of 3.2 cc. (1.4 mols per mol) of benzoyl chloride diluted to 18 cc. volume with chloroform, was added slowly over a period of about 20 minutes. The mixture was allowed to stand at room temperature for 4 hours and 5 cc. of water were added. After standing another half hour, ice water and chloroform were added and the extracts were treated as for the substance described above. The washed, dried chloroform extracts were concentrated under reduced pressure to a thick syrup and this was dissolved in a little warm ether. On cooling this solution, the product immediately crystallized. Yield 9 gm. The product was recrystallized by dissolving in warm ether, cooling, and adding pentane. It also crystallized readily from 75 per cent alcohol. The composition corresponded to a tribenzoyl methylhexoside.

4.349 mg. substance: 10.610 mg. CO_2 and 2.090 mg. H_2O
6.845 " " : 3.031 " AgI
$C_{28}H_{26}O_9$. Calculated. C 66.38, H 5.18, OCH_3 6.12
506.2 Found. " 66.52, " 5.37, " 5.84

The melting point was 144.5–145.5° and the rotation, in chloroform, was $[\alpha]_D^{20} = \frac{+3.28° \times 100}{2 \times 2.0} = +82.0°$.

2,3,4-Tribenzoyl-β-methylglucoside has been described, but only as a syrup,[10] and no rotation was reported. 2,3,4,6-Tetrabenzoyl-β-methylglucoside[11] melts at 160–162° and has a rotation, in chloroform, of $[\alpha]_D^{20} = +30.99°$.

4-Acetyl-2,3,6-Tribenzoyl-β-Methylglucoside—2 gm. of the tribenzoyl-β-methylglucoside were dissolved in 10 cc. of dry pyridine and 0.6 cc. (1½ mols) of acetic anhydride were added. The mixture warmed slightly. After standing 24 hours at room temperature, 2 cc. of water were added. After ½ hour, more water was added and the mixture was three times extracted with chloroform, and worked up as for the tribenzoyl compound above. After concentrating the washed, dried extract to a thick syrup, ether was poured into the flask and the product crystallized instantly. The ether was evaporated off and the product was recrystallized from methyl alcohol. After recrystallization from the same solvent, the yield was 1.2 gm. and an additional 0.6 gm. of less pure material was recovered from the mother liquors.

The composition of the pure product corresponded to an acetyl tribenzoyl methylhexoside.

4.653 mg. substance: 11.235 mg. CO_2 and 2.090 mg. H_2O
6.511 " " : 2.885 " AgI
$C_{30}H_{28}O_{10}$. Calculated. C 65.67, H 5.15, OCH_3 5.66
548.2 Found. " 65.84, " 5.02, " 5.85

The melting point was 156–157° and the rotation, in chloroform, was

$$[\alpha]_D^{20} = \frac{+3.84° \times 100}{2 \times 2.0} = +96.0°.$$

6-Acetyl-2,3,4-tribenzoyl-β-methylglucoside has been described.[10,12] The melting point is 150–151° and the rotation, in chloroform, is $[\alpha]_D^{20} = -6.5°$.

4-p-Tolylsulfo-2,3,6-Tribenzoyl-β-Methylglucoside—10 gm. of pure tribenzoyl-β-methylglucoside were dissolved in 50 cc. of dry pyridine, 4.1 gm. (1.1 mols) of *p*-toluenesulfonchloride were added, the mixture was shaken until the chloride dissolved and was then kept at 50° for 24 hours. Water was added just to turbidity, and after ½ hour at room temperature, more water was added. The mixture was extracted three times with chloroform and the extracts were worked up as for the compounds above. The washed, dried extracts were concentrated to a syrup which crystallized immediately upon addition of ether. From the ether solution additional material was recovered, making the total yield of crude product 6.6 gm. It was recrystallized by dissolving in warm ethyl acetate, adding an equal volume of hexane, and cooling. After 2 hours in the ice box, the crystals were filtered off, washed with ethyl acetate-hexane, and air-dried at 50°. Material twice crystallized in this manner had a composition which corresponded to a tolylsulfo tribenzoyl methylhexoside.

---

[10] Josephson, K., *Ber. chem. Ges.*, **62**, 313 (1929).

[11] Fischer, E., and Helferich, B., *Ann. Chem.*, **383**, 90 (1911).

[12] Bergmann, M., and Koch, F. K. V., *Ber. chem. Ges.*, **62**, 311 (1929).

4.900 mg. substance: 11.475 mg. CO_2 and 2.218 mg. H_2O
0.1530 gm. " : 0.0576 gm. AgI
13.640 mg. " : 4.699 mg. $BaSO_4$

$C_{35}H_{32}O_{11}S$.	Calculated.	C 63.60, H 4.92, OCH_3 4.69, S 4.85
660.3	Found.	" 63.87, " 5.06, " 4.96, " 4.74

The product melted and decomposed at 191–192° and had a rotation, in chloroform, of $[\alpha]_D^{20} = \frac{+1.72^\circ \times 100}{2 \times 2.0} = +43.0^\circ$.

Experiments designed to remove the benzoyl groups, while leaving the tolylsulfo group intact, have thus far been unsuccessful.

4-Methyl-2,3,6-Tribenzoyl-β-Methylglucoside—10 gm. of the pure tribenzoyl-β-methylglucoside were dissolved in 40 cc. of methyl iodide and methylated by the Purdie method, 16 gm. of silver oxide being added in four equal portions at ½ hour intervals. After the methylation, the product was isolated by extraction with boiling chloroform, and the filtered extracts were concentrated under reduced pressure to a syrup. This was redissolved in methyl iodide and remethylated exactly as before. The final product was a syrup which did not crystallize. A small portion was dried to a solid transparent mass over phosphorus pentoxide, powdered, and redried under greatly reduced pressure. The analysis indicated it to be incompletely methylated.

6.321 mg. substance: 5.251 mg. AgI

$C_{29}H_{28}O_9$.	Calculated.	OCH_3 11.92
520.2	Found.	" 10.96

The rotation, in chloroform, was $[\alpha]_D^{21} = \frac{+2.34^\circ \times 100}{2 \times 2.0} = +58.5^\circ$.

4-Methyl-2,3,6-Triacetyl-β-Methylglucoside—The syrup obtained above was dissolved in 50 cc. of methyl alcohol. 0.3 cc. of a 2.4 N solution of barium oxide in methyl alcohol was added and the mixture was kept at room temperature for 1 hour and then at 50° for 1 hour. An additional 0.3 cc. of the barium oxide solution was added and the mixture was refluxed on the steam bath for an hour. It was then concentrated under reduced pressure to about 10 cc. volume, ether was added, and the flocculent precipitate which formed was filtered off and discarded. The filtrate was concentrated under reduced pressure to a syrup which was dissolved in 75 cc. of pyridine. 6.5 cc. of acetic anhydride (3.3 mols) were added and the mixture was allowed to stand overnight at room temperature. Water was added and after $\frac{1}{2}$ hour the product was isolated by chloroform extraction and worked up as for the substances above. After concentrating the washed, dried chloroform extract to a thick syrup, it was dissolved in ether and cooled. Pentane was added just to turbidity, and the mixture was allowed to stand in the ice box. The product crystallized after 2 days. Yield 2.6 gm. It was recrystallized once from ether by adding pentane, and then once from ether alone. The composition corresponded to a methyl triacetyl methylhexoside.

4.105 mg. substance: 7.594 mg. CO_2 and 2.501 mg. H_2O
4.550 " " : 6.435 " AgI
$C_{14}H_{22}O_9$. Calculated. C 50.27, H 6.63, OCH_3 18.55
334.2 Found. " 50.44, " 6.81, " 18.66

The rotation, in chloroform, was $[\alpha]_D^{20} = \frac{-1.31° \times 100}{2 \times 2.0} = -32.8°$. The melting point was 107–108° and the melting point of an intimate mixture with the 4-methyl triacetyl-β-methylglucoside described below was the same, 107–108°.

4-Methyl-2,3,6-Triacetyl-β-Methylglucoside—The "trimethyl" dibenzylmercaptoglucose of Pacsu[2] was twice recrystallized. The methoxyl content (7.25 per cent) agreed with Schinle's[1] conclusion that the substance is a monomethyl dibenzylmercaptoglucose (methoxyl 7.31 per cent). It was hydrolyzed as described by Pacsu to the free methyl glucose, obtained as a syrup. This was converted to the tetracetate with pyridine and acetic anhydride and the syrupy product was converted to the acetobromo compound with a saturated solution of hydrogen bromide in glacial acetic acid in the usual manner. No attempt was made to crystallize the bromo compound; instead, it was dissolved in dry methyl alcohol and shaken with dry silver oxide until the test for halide was negative. The mixture was filtered with charcoal and concentrated under reduced pressure to a small volume. The product crystallized on standing overnight in the ice box. It was filtered off, washed with a little methyl alcohol, and dried. For recrystallization it was dissolved in ether and cooled, and pentane was added just to turbidity. After standing overnight, the substance was filtered off, washed with the same solvents, and recrystallized from ether. The product thus obtained was air-dried. The composition corresponded to a methyl triacetyl methylhexoside.

4.765 mg. substance: 8.830 mg. CO_2 and 2.800 mg. H_2O
3.115 " " : 4.301 " AgI

$C_{14}H_{22}O_9$.	Calculated.	C 50.27,	H 6.63,	OCH_3	18.55
334.2	Found.	" 50.53,	" 6.57,	"	18.22

The rotation, in chloroform, was $[\alpha]_D^{20} = \frac{-1.34° \times 100}{2 \times 2.0} = -33.5°$. The melting point was 107–108° and a mixed melting point with the methyl triacetyl methylglucoside described above was the same, 107–108°.

2,3-Diacetyl-β-Methylglucoside—Diacetyl benzylidene-β-methylglucoside was prepared by acetylation of benzylidene-β-methylglucoside with acetic anhydride and sodium acetate in the usual manner. It was then deacetylated in a manner similar to that described for the corresponding benzoyl compound. 0.025 N hydrochloric acid, as used by Oldham and Rutherford[6] was found to give better results than the 0.1 N which we used initially. The hydrolyzing mixture was refluxed for 5 hours, however, instead of 4. For isolation, the hydrochloric acid was removed by shaking the cooled solution with silver oxide. The mixture was filtered and the filtrate was concentrated under reduced pressure until two layers formed, extracted three times with pentane in a separatory funnel, and filtered with charcoal. The filtrate was concentrated under reduced pressure to a thick syrup.

2,3-Diacetyl-4,6-Dibenzoyl-β-Methylglucoside—The syrup, obtained as above from 8.2 gm. of diacetyl benzylidene-β-methylglucoside was distilled with pyridine and benzene for some time to remove all water and then dissolved in 50 cc. of dry pyridine. 7.7 cc. (3 mols) of benzoyl chloride were added and the mixture was allowed to stand overnight at room temperature. 5 cc. of water were added, and after ½ hour, ice water and chloroform. The product was extracted three times with chloroform and worked up as described for the derivatives above. The washed, dried chloroform extracts were concentrated under reduced pressure to a syrup, and ether was added. Crystallization started immediately. After 3 or 4 hours in the ice box, the product was filtered off, washed with ether, and dried. The yield was 6.1 gm. and an additional 1.6 gm. was obtained by working up the mother liquor. The product was three times recrystallized from 10 to 12 parts of absolute alcohol. Its composition corresponded to a diacetyl dibenzoyl methylhexoside.

4.476 mg. substance: 10.115 mg. CO_2 and 2.200 mg. H_2O
6.187 " " : 3.050 " AgI

$C_{25}H_{26}O_{10}$.	Calculated.	C 61.70, H 5.39, OCH_3 6.38
486.2	Found.	" 61.63, " 5.50, " 6.51

The substance melted at 164–165° and had a rotation, in chloroform of $[\alpha]_D^{20} = \frac{-0.23° \times 100}{2 \times 2.0} = -5.8°$.

2,3,6-Triacetyl-β-Methylglucoside—The syrup from the hydrolysis of 25 gm. of diacetyl benzylidene-β-methylglucoside was distilled with benzene and pyridine to remove remaining water and was then dissolved in 50 cc. of dry pyridine. The solution was stirred mechanically and cooled in running water. A solution of 7.5 cc. of acetic anhydride (1.1 mols) in 30 cc. of chloroform was added drop by drop over a period of about 20 minutes. The mixture was then allowed to stand overnight at room temperature and 5 cc. of water were added. After ½ hour the product was isolated with chloroform as described above. The washed, dried chloroform extracts were concentrated under reduced pressure to a thick syrup which was dissolved in a little warm ether. On standing in the ice box for a few hours, crystals appeared, and on stirring with a rod, the product crystallized promptly. It was filtered off, washed with ether, and dried. Yield 11 gm. Further amounts were obtained from the mother liquors only with difficulty.

The product was dissolved in boiling ether and concentrated to about 50 cc. The solution was cooled, an equal volume of pentane was added, and the product immediately crystallized. It was then twice recrystallized by dissolving in boiling ether, concentrating, and cooling. The composition of the product corresponded to a triacetyl methylhexoside.

4.799 mg. substance: 8.557 mg. CO_2 and 2.741 mg. H_2O
4.751 " " : 3.580 " AgI
$C_{13}H_{20}O_9$. Calculated. C 48.70, H 6.29, OCH_3 9.68
320.2 Found. " 48.62, " 6.38, " 9.94

In an acetyl determination, 0.100 gm. required 9.37 cc. of 0.1 N sodium hydroxide, while the theoretical value is 9.44 cc.

The melting point of the pure product was 113.5–114.5° and the rotation, in chloroform, was $[\alpha]_D^{20} = \frac{-2.36° \times 100}{2 \times 2.0} = -59.0°$. Helferich, Bredereck, and Schneidmüller[4] give 114–115° as the melting point of their 2,3,6-triacetyl-β-methylglucoside and −64.9° as its rotation in chloroform. 2,3,4-Triacetyl-β-methylglucoside[4] melts at 134° and has a rotation, in chloroform, of −18.8°.

4-Benzoyl-2,3,6-Triacetyl-β-Methylglucoside—1.5 gm. of the triacetyl-β-methylglucoside, obtained above, were dissolved in 15 cc. of dry pyridine, and 1.1 cc. of benzoyl chloride (2.2 mols) were added. After standing overnight at room temperature, 2 cc. of water were added, and after another half hour, more water. The product was isolated by chloroform extraction and worked up as for the products above. The washed, dried chloroform extract was concentrated under reduced pressure to a thick syrup which was dissolved in ether. After cooling, pentane was added just to turbidity. After a few days in the ice box, a few crystals formed, and on scratching with a glass rod the product crystallized immediately. The total yield was 1.7 gm. The product was crystallized from ether by adding pentane, and then once from ether alone. The composition corresponded to a benzoyl triacetyl methylhexoside.

5.005 mg. substance: 10.395 mg. CO_2 and 2.520 mg. H_2O
6.085 " " : 3.358 " AgI
$C_{20}H_{24}O_{10}$. Calculated. C 56.58, H 5.70, OCH_3 7.31
424.2 Found. " 56.63, " 5.63, " 7.28

The melting point was 98–99° and the rotation, in chloroform, was $[\alpha]_D^{20} = \frac{-2.44° \times 100}{2 \times 2.0} = -61.0°$.

Ohle and Spencker[13] have described 6-benzoyl-2,3,4-triacetyl-β-methylglucoside, melting point 127° and rotation +15.15°.

4-p-Tolylsulfo-2,3,6-Triacetyl-β-Methylglucoside—1.5 gm. of the triacetyl-β-methylglucoside were dissolved in 15 cc. of dry pyridine and 1.8 gm. (2 mols) of *p*-toluenesulfonchloride were added. The mixture was shaken until all the chloride dissolved and was then allowed to stand at room temperature overnight. 2 cc. of water were added, and after ½ hour, more water. The product was extracted with chloroform and worked up as described for the compounds above. The washed, dried chloroform extract was concentrated under reduced pressure to a thick syrup, which was dissolved in a little ether. On adding pentane to turbidity, the product crystallized immediately. The first crop weighed 1.6 gm. and the mother liquors were not worked up for further quantities. It was twice recrystallized from absolute alcohol. The composition corresponded to a tolylsulfo triacetyl methylhexoside.

4.821 mg. substance: 9.007 mg. CO_2 and 2.290 mg. H_2O
4.792 " " : 2.455 " AgI
11.880 " " : 5.702 " $BaSO_4$

$C_{20}H_{26}O_{11}S$.	Calculated.	C 50.61, H 5.53, OCH_3 6.54, S 6.76
474.2	Found.	" 50.94, " 5.31, " 6.76, " 6.59

The melting point was 116–117° and the rotation in chloroform was $[\alpha]_D^{20} = \frac{-1.60° \times 100}{2 \times 2.0} = -40.0°$ and in pyridine $[\alpha]_D^{20} = \frac{-1.18° \times 100}{2 \times 2.0} = -29.5°$. Helferich, Bredereck, and Schneidmüller[4] give 118° as the melting point, and −29.7° as the rotation in pyridine, of the *p*-tolylsulfo derivative of their 2,3,6-triacetyl-β-methylglucoside. The 6-*p*-tolylsulfo-2,3,4-triacetyl-β-methylglucoside[4,13] has a melting point of 171° and rotations in pyridine and chloroform respectively of +33.1° and +12.03°.

---

[13] Ohle, H., and Spencker, K., *Ber. chem. Ges.*, **59**, 1836 (1926).

Addendum—While this paper was in press we found a very recent article by Brigl and Grüner (*Ann. Chem.*, **495**, 60 (1932)) in which 2,3-dibenzoyl-4,6-diacetyl-β-methylglucoside was prepared by the same method which we employed. Almost identical properties were reported (m. p. 132–133° and $[\alpha]_D^{20} = +79.3$ $CHCl_3$). These authors assign a structure of 2,6-dibenzoyl to the compounds of Josephson.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, October, 1932,
Vol. 98, No. 1, pp. 1-7]

MAXIMUM ROTATIONS AND CORRELATION OF DISUBSTITUTED ACETIC ACIDS CONTAINING A METHYL GROUP

By P. A. LEVENE AND R. E. MARKER

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 20, 1932)

In a recent article[1] the rotations of members of homologous series of disubstituted propionic, butyric, and valeric acids were compared (each containing the substituting alkyl groups on the terminal carbon atom, and one of the groups attached to the asymmetric carbon atom being a methyl group). The starting member was levo-3-methyl-3-ethylpropionic acid. The values of the rotations of the members of the homologous acids progressively increased towards the right. Similar changes were observed in the members of the derivatives of the configurationally related butyric and valeric acids and in the configurationally related propyl carbinols and propyl chlorides derived from the homologous members of the levo-3-methyl-3-ethylpropionic acids. These observations seemed to point to the possibility of formulating a general conclusion to the effect that the direction of the change in rotations of consecutive members is the same for all configurationally related homologous series. At the time of that publication[1] the maximum values of the rotations of the members of the homologous series of the corresponding substituted acetic acids were not yet known. Hence, several members of this series have been resolved until further increase in their rotation was not obtainable by the methods available at present. The results are given in Table I, in which, for comparison, the rotations of the corresponding members of the acids previously studied are also given.

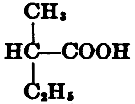
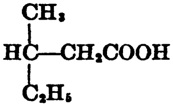
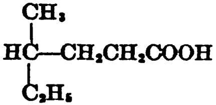
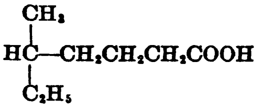
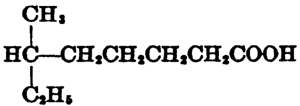
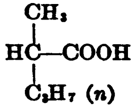
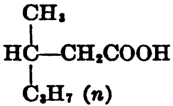
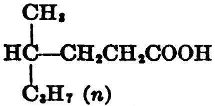
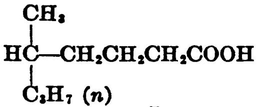
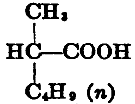
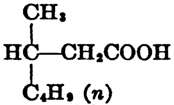
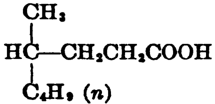
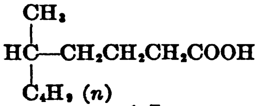
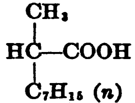
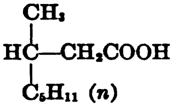
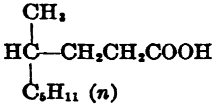
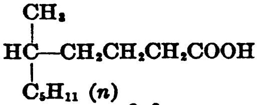
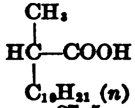
From Table I it may be seen that the values of the rotation of

---

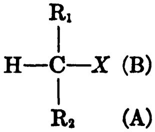
[1] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **95**, 153 (1932).

TABLE I

Maximum Molecular Rotations of Configurationally Related Aliphatic Acids Containing a Methyl Group on the Asymmetric Carbon Atom, $[M]_D^{25}$ *(Homogeneous)*

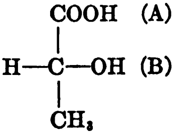
 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}COOH}\\ \mid\\ \mathrm{C_2H_5}\end{array}$ −18.0	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2COOH}\\ \mid\\ \mathrm{C_2H_5}\end{array}$ −10.4	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2COOH}\\ \mid\\ \mathrm{C_2H_5}\end{array}$ −13.6	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2CH_2COOH}\\ \mid\\ \mathrm{C_2H_5}\end{array}$ −11.1	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2CH_2CH_2COOH}\\ \mid\\ \mathrm{C_2H_5}\end{array}$ −12.2
 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}COOH}\\ \mid\\ \mathrm{C_3H_7}\ (n)\end{array}$ −21.4	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2COOH}\\ \mid\\ \mathrm{C_3H_7}\ (n)\end{array}$ +3.6	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2COOH}\\ \mid\\ \mathrm{C_3H_7}\ (n)\end{array}$ −6.9	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2CH_2COOH}\\ \mid\\ \mathrm{C_3H_7}\ (n)\end{array}$ −3.7	
 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}COOH}\\ \mid\\ \mathrm{C_4H_9}\ (n)\end{array}$ −24.3	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2COOH}\\ \mid\\ \mathrm{C_4H_9}\ (n)\end{array}$ +6.1	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2COOH}\\ \mid\\ \mathrm{C_4H_9}\ (n)\end{array}$ −4.1	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2CH_2COOH}\\ \mid\\ \mathrm{C_4H_9}\ (n)\end{array}$ −1.7	
 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}COOH}\\ \mid\\ \mathrm{C_7H_{15}}\ (n)\end{array}$ −27.3	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2COOH}\\ \mid\\ \mathrm{C_5H_{11}}\ (n)\end{array}$ +8.1	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2COOH}\\ \mid\\ \mathrm{C_5H_{11}}\ (n)\end{array}$ −1.9	 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}CH_2CH_2CH_2COOH}\\ \mid\\ \mathrm{C_5H_{11}}\ (n)\end{array}$ −0.6	
 $\begin{array}{l}\mathrm{CH_3}\\ \mid\\ \mathrm{HC{-}COOH}\\ \mid\\ \mathrm{C_{10}H_{21}}\ (n)\end{array}$ −27.5				

the members of the substituted acetic acid series progressively increase to the left, contrary to the conduct of the members of the other series. Thus the rule of the correlation of the configurations of homologous series by the direction of the change of rotation of consecutive members is limited to those series in which the functional group is attached to a carbon atom located at a distance from the asymmetric carbon atom. In fact, the older observations on the α-thiol and α-sulfo acids already pointed towards the possibility that in two configurationally related homologous series,[1] the change in rotation of consecutive members may have a different direction when the series differ in the character of a functional group attached directly to the asymmetric carbon atom. From theoretical considerations, such a state of affairs is readily understandable. If, as in the previous communication, the contributions of the two groups are designated by the letters A and B,



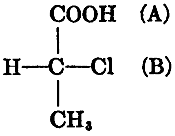
$$\begin{array}{lll} & R_1 & \\ & | & \\ H- & C & -X\ \ (B) \\ & | & \\ & R_2 & \quad\ \ (A) \end{array}$$

and if it is assumed that in consecutive members both values increase numerically, then in the visible part of the spectrum the change of the direction of rotation of the consecutive members will depend upon whether A or B furnishes the higher contribution. Thus, if we take the case of two acids



$$\begin{array}{lll} & COOH & \ (A) \\ & | & \\ H- & C & -OH\ \ (B) \\ & | & \\ & CH_3 & \end{array}$$

I



$$\begin{array}{lll} & COOH & \ (A) \\ & | & \\ H- & C & -Cl\ \ (B) \\ & | & \\ & CH_3 & \end{array}$$

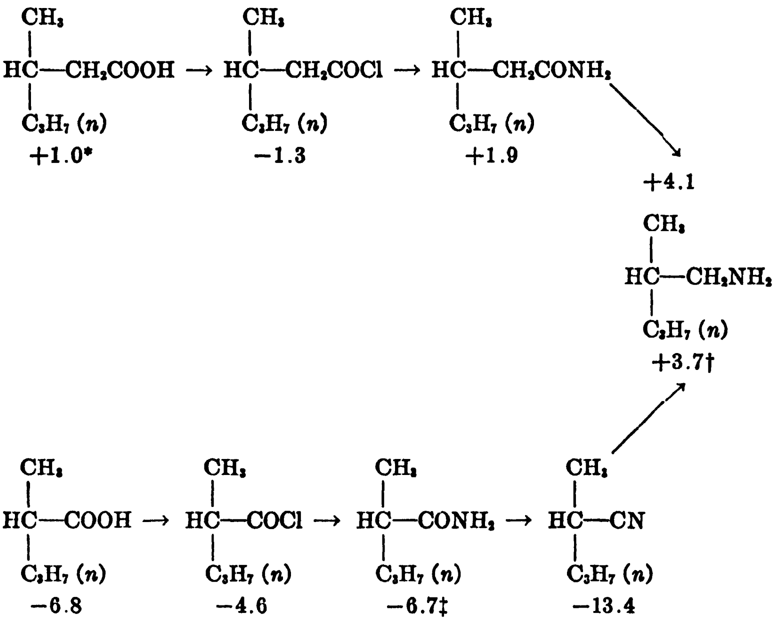
II

and assume that the numerical values of each increase in consecutive members, then the direction of the change will depend upon whether A or B furnishes the major contribution. Thus, assuming that in both I and II the —COOH group has a dextrorotatory and B a levorotatory contribution and that in I the major contribution is furnished by A, and in II by B, the changes in I may be to the right and in II to the left.

In addition to the data on the maximum rotation of the members of the disubstituted acetic acids, the present article contains additional evidence towards the correlation of the configurations of the series of disubstituted acetic and propionic acids.

TABLE II

Correlation of Methyl-n-Propylpropionic Acid to Methyl-n-Propylacetic Acid, $[M]_D^{25}$ (Homogeneous) (Not Maximum Rotations)



$$\begin{tikzcd}
\begin{array}{l}CH_3\\ \vert\\ HC—CH_2COOH\\ \vert\\ C_3H_7\ (n)\\ +1.0^*\end{array} \arrow[r] & \begin{array}{l}CH_3\\ \vert\\ HC—CH_2COCl\\ \vert\\ C_3H_7\ (n)\\ -1.3\end{array} \arrow[r] & \begin{array}{l}CH_3\\ \vert\\ HC—CH_2CONH_2\\ \vert\\ C_3H_7\ (n)\\ +1.9\end{array} \arrow[dr] & \\
 & & & \begin{array}{l}+4.1\\ CH_3\\ \vert\\ HC—CH_2NH_2\\ \vert\\ C_3H_7\ (n)\\ +3.7\dagger\end{array} \\
\begin{array}{l}CH_3\\ \vert\\ HC—COOH\\ \vert\\ C_3H_7\ (n)\\ -6.8\end{array} \arrow[r] & \begin{array}{l}CH_3\\ \vert\\ HC—COCl\\ \vert\\ C_3H_7\ (n)\\ -4.6\end{array} \arrow[r] & \begin{array}{l}CH_3\\ \vert\\ HC—CONH_2\\ \vert\\ C_3H_7\ (n)\\ -6.7\ddagger\end{array} \arrow[r] & \begin{array}{l}CH_3\\ \vert\\ HC—CN\\ \vert\\ C_3H_7\ (n)\\ -13.4\end{array} \arrow[u]
\end{tikzcd}$$

---

* For intermediate steps, see Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91,** 77 (1931).

† Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **84,** 571 (1929).

‡ In alcohol.

The evidence thus far furnished is the following: (1) 3-Methyl-3-ethylpropionic acid has been correlated with the corresponding acetic acid by the synthesis of 4-methyl-4-ethylbutyric acid from 2-methyl-2-ethyl-1-bromoethane to which then was correlated 3-methyl-3-ethylpropionic acid. (2) 3-Methyl-3-*n*-butylpropionic acid has been correlated with the corresponding acetic acid by the 2-methyl-2-*n*-butyl-1-aminoethanes derived from them. (3) 3-Methyl-3-*n*-propylpropionic acid has now been correlated with the acetic acid series by the set of reactions given in Table II.

Thus, three members of the acetic acid series have been correlated by direct chemical methods with the corresponding members of the propionic acid series. Hence, it is justified to conclude that the higher members of the two series given in the first and second columns of Table I also are configurationally related. It thus follows that the increase in rotation of the consecutive members follows a different direction in each of these two series. Work is now in progress to determine which group in each series is responsible for the direction of rotation in the visible part of the spectrum.

EXPERIMENTAL

Methylethylacetic Acid—The inactive acid was prepared by the malonic ester synthesis. This was resolved by means of its brucine salt from water as described by Marckwald.[2] The maximum value of the rotation was the same as that reported by him, $[M]_D^{25} = +18°$ (homogeneous).

Levo-Methyl-n-Propylacetic Acid—The inactive acid was dissolved in boiling acetone and an equivalent weight of quinine was added. After eighteen recrystallizations, the rotation of the free acid reached a constant value. B.p. 96° at 15 mm. $D\frac{25}{4} = 0.920$. $n_D^{25} = 1.4117$.

$$[\alpha]_D^{25} = \frac{-16.90°}{1 \times 0.920} = -18.4°; [M]_D^{25} = -21.4° \text{ (homogeneous)}$$

4.025 mg. substance: 9.200 mg. CO_2 and 3.835 mg. H_2O

$C_6H_{12}O_2$.	Calculated.	C 62.0, H 10.4
116.1	Found.	" 62.3, " 10.7

0.063 gm. substance: 5.45 cc. 0.1 N NaOH (titration, phenolphthalein). Calculated, 5.43 cc.

Levo-Methyl-n-Butylacetic Acid—The inactive acid was dissolved in hot 66 per cent acetone and an equivalent weight of cinchonidine added. After eight recrystallizations from 66 per cent acetone the rotation of the free acid reached a constant value. B.p. 105° at 5 mm. $D\frac{25}{4} = 0.909$. $n_D^{25} = 1.4189$.

---

[2] Marckwald, W., *Ber. chem. Ges.*, **37**, 1045, 1048 (1904).

$$[\alpha]_D^{25} = \frac{-17.0°}{1 \times 0.909} = -18.7°; [M]_D^{25} = -24.3° \text{ (homogeneous)}$$

3.980 mg. substance: 9.490 mg. CO_2 and 3.920 mg. H_2O
$C_7H_{14}O_2$. Calculated. C 64.6, H 10.8
130.1 Found. " 65.0, " 11.0
0.1030 gm. substance: 7.86 cc. 0.1 N NaOH (titration, phenolphthalein). Calculated, 7.92 cc.

Levo-Methyl-n-Heptylacetic Acid—The inactive acid was dissolved in boiling acetone and an equivalent weight of cinchonidine was added. After twelve recrystallizations the rotation of the free acid reached a constant value. B.p. 115° at 1 mm. $D\frac{23}{4} = 0.895$. $n_D^{25} = 1.4312$.

$$[\alpha]_D^{23} = \frac{-14.25°}{1 \times 0.895} = -15.9°; [M]_D^{23} = -27.3° \text{ (homogeneous)}$$

5.430 mg. substance: 14.020 mg. CO_2 and 5.770 mg. H_2O
$C_{10}H_{20}O_2$. Calculated. C 69.7, H 11.6
172.16 Found. " 70.4, " 11.8
0.146 gm. substance: 8.55 cc. 0.1 N NaOH (titration, phenolphthalein). Calculated, 8.49 cc.

Dextro-Methyl-n-Decylacetic Acid—The inactive acid was dissolved in boiling acetone and an equivalent weight of cinchonidine was added. It was allowed to crystallize in a refrigerator at −10°. After ten recrystallizations there was no change in the rotation of the free acid. The total was treated with hydrochloric acid, extracted with ether, purified through its sodium salt, then distilled. B.p. 150° at 1 mm. $D\frac{25}{4} = 0.884$. $n_D^{25} = 1.4395$.

$$[\alpha]_D^{25} = \frac{+11.35°}{1 \times 0.884} = +12.85°; [M]_D^{25} = +27.5° \text{ (homogeneous)}$$

4.116 mg. substance: 11.085 mg. CO_2 and 4.555 mg. H_2O
$C_{13}H_{26}O_2$. Calculated. C 72.8, H 12.2
214.2 Found. " 73.4, " 12.4
0.137 gm. substance: 6.46 cc. 0.1 N NaOH (titration, phenolphthalein). Calculated, 6.40 cc.

Dextro-2-Methyl-1-Aminopentane—25 gm. of 2-propylbutyric acid-4, $[M]_D^{25} = +1.01°$ (homogeneous), were converted into the amide as previously described.[3]

18 gm. of bromine were dissolved in 60 gm. of potassium hydroxide in 1 liter of water. This solution was poured onto 20 gm. of amide. The mixture was heated on the steam bath for 1 hour, then steam-distilled. The distillate was acidified with hydrochloric acid, extracted with ether, the concentrate then made alkaline, and the amine extracted with ether. B.p. 64° at 90 mm. $D\frac{25}{4} = 0.763$.

$$[\alpha]_D^{25} = \frac{+3.12°}{1 \times 0.763} = +4.09°; [M]_D^{25} = +4.13° \text{ (homogeneous)}$$

3.280 mg. substance: 8.550 mg. CO_2 and 4.395 mg. H_2O

$C_6H_{15}N$. Calculated. C 71.2, H 14.9
101.12 Found. " 71.1, " 15.0

---

[3] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 77 (1931).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, October, 1932,
Vol. 98, No. 1, pp. 9–16]

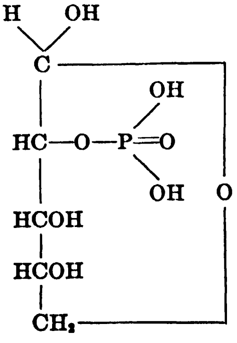
THE RIBOSEPHOSPHORIC ACID FROM XANTHYLIC ACID. II

BY P. A. LEVENE AND STANTON A. HARRIS

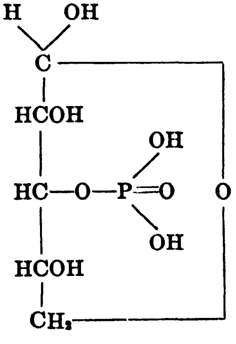
(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 20, 1932)

In a previous communication[1] it was shown that the ribosephosphoric acid obtained from xanthylic acid and hence from guanylic acid was different from 5-ribosephosphoric acid derived from inosinic acid. Furthermore, it was found by Levene and Tipson[2] that guanosine and therefore xanthosine possess the furanoside structure. Thus only two structures should be considered for the new ribosephosphoric acid; namely, the one with the phosphoric acid residue in position (2) and that in position (3). In the present communication conclusive evidence is furnished in favor of the latter structure (II).



I



II

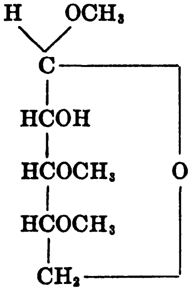
The methylation method was first chosen for the purpose of establishing the structure of the new ribosephosphoric acid. After the methylation was accomplished, two alternative methods *a priori* were available for establishing the position of the phosphoric acid.

---

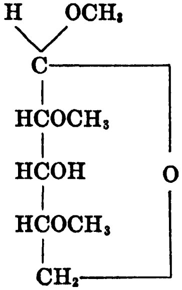
[1] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **95**, 755 (1932).

[2] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **97**, 491 (1932).

The first method was the removal of the phosphoric acid with subsequent isolation of the methylated normal riboside. The structure of the dimethylriboside, depending upon the position of the phosphoric acid residue, should be either III or IV. Only substance III can yield an optically active dimethyltrihydroxyglutaric acid.

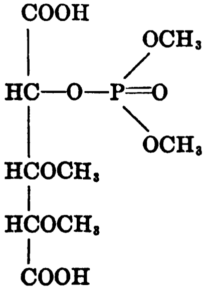


III

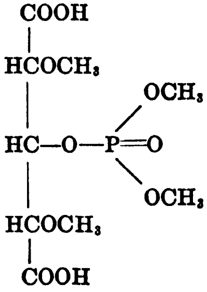


IV

The second method was the oxidation of the methylated ribosephosphoric acid to the corresponding phosphorylated trihydroxyglutaric acid. Only one (V) of the two possible acids could be optically active.



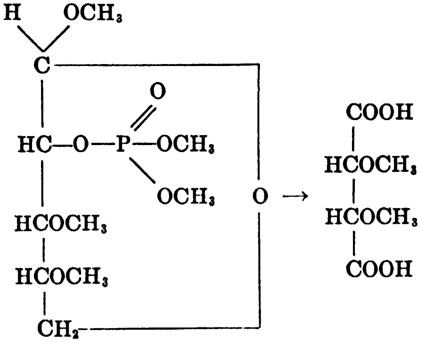
V



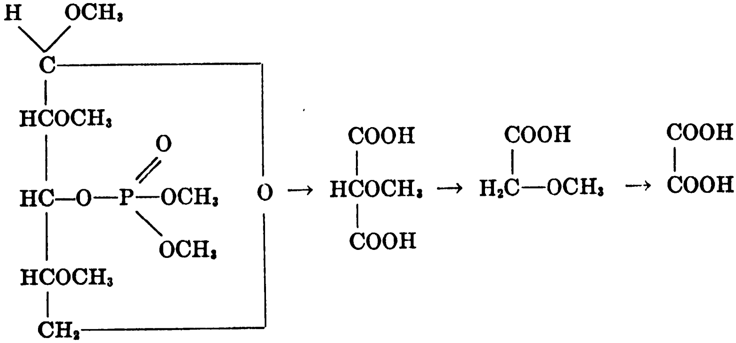
VI

However, neither of these two methods yielded satisfactory results. The failure of the first was due to the unexpected resistance of the phosphoric acid residue towards the hydrolytic action of hydrogen or hydroxyl ions, so that it was not possible to obtain a satisfactory yield of the methylated sugar.

The second method was also found unsatisfactory. By the milder oxidation with nitric acid, the expected dimethyltrihydroxyglutaric acid was not obtained. More rigorous oxidation was then attempted. It was expected that this procedure might lead to simultaneous dephosphorylation and oxidation of the dimethylribose. Either mesodimethyltartaric acid, in case of VII, or methoxymalonic acid, in case of VIII, should be formed. The latter substance would be expected to form methylglycolic acid, which perhaps could be oxidized into oxalic acid. Indeed, oxalic acid was the only substance which could be identified among the



VII

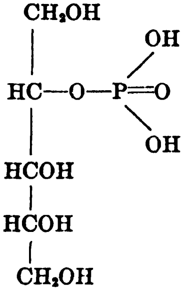


VIII

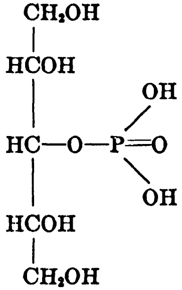
products of the reaction. Thus, by this method indirect evidence was secured in support of II. The evidence, however, was not conclusive.

In view of the unsatisfactory results obtained by both of these methods, it was decided to resort to the reduction of the *d*-ribosephosphoric acid to the corresponding *d*-ribitolphosphoric acid. Again one of two alternative possibilities was envisaged, depending upon the structure of the new ribosephosphoric acid.

Substance I should lead to an optically active ribitolphosphoric acid, IX, whereas substance II should form an optically inactive derivative, X.



IX



X

Two methods available for the reaction were: the reduction by means of sodium amalgam and the catalytic reduction by hydrogen in the presence of platinic oxide (reagent of Roger Adams). In a preliminary way, both methods were tested on hexose-3-phosphate. In this case more satisfactory results were obtained by the amalgam reduction. On the other hand, in the case of *d*-ribosephosphoric acid, the catalytic method gave a better yield of a purer material which proved to be the inactive ribitolphosphoric acid which consequently had the structure X. The optical activity was tested both in aqueous solution and in a solution of sodium borate. Thus, the structure of the new ribosephosphoric acid is definitely established as *d*-ribose-3-phosphoric acid (II).

EXPERIMENTAL

Methylation of Ribosephosphoric Acid—A solution containing 25 gm. of barium ribosephosphate was treated with dilute sulfuric acid until no test for barium or sulfate was obtainable. This solution was then shaken with a slight excess of silver oxide (16.6 gm.). The silver salt was precipitated by adding an equal volume of methyl alcohol. The precipitate was washed with methyl alcohol and ether and dried to constant weight over phosphorus pentoxide in a vacuum desiccator. The yield of silver salt was 22.5 gm. or 72 per cent of the theoretical.

25 gm. of the dry silver salt were treated with 960 cc. of reagent methyl alcohol containing 8.5 gm. of hydrogen chloride. This gave a solution of ribosephosphoric acid containing approximately 0.5 per cent of hydrogen chloride. This solution was allowed to stand for 9 days at 50° and 4 days at 58°, after which it was filtered and neutralized with dry silver oxide. No appreciable amount of phosphoric acid was liberated at 50°, but after 4 additional days at 58°, it was found that 17 per cent of the phosphorus had been hydrolyzed. Enough silver oxide was used to form the silver salt of the phospho-*n*-methylriboside. The solution containing the precipitated silver salt was evaporated to one-half its volume. It was then treated with a total of 23 cc. of methyl iodide and heated on a steam bath for 2 hours. The silver halides were removed by filtration and thoroughly washed with methyl alcohol. The filtrate, containing the methyl ester of the phospho-*n*-methylriboside, was evaporated to a thick syrup. This syrup was taken up in 35 cc. of methyl iodide and 15 cc. of methyl alcohol. Methylation started spontaneously on the addition of silver oxide. The material was methylated and remethylated once by the Purdie process.

After completion of the methylation the silver oxide and silver iodide were filtered off and extracted five times with boiling chloroform. The chloroform solution was dried over sodium sulfate and concentrated to a syrup under reduced pressure. On distillation a forerun was obtained, distilling at 65° at 0.5 mm. pressure and weighing 3.5 gm. Judging from analytical data, it consisted of methylated phosphoric acid and methylated ribose. The remainder distilled at 128–133° under 0.1 mm. pressure. The yield was 5.1 gm. The refractive index of the methylated product from the higher boiling fraction was $n_D^{22} = 1.4498$.

The specific rotation was as follows:

$$[\alpha]_D^{28} = \frac{-1.765° \times 100}{5.13 \times 2} = -17.2°$$

The substance had the composition of dimethylphosphodimethyl-*n*-methylriboside.

5.480 mg. substance: 8.109 mg. CO_2 and 3.520 mg. H_2O
5.554 " " 21.800 " AgI
4.372 " " 31.240 " ammonium phosphomolybdate
$C_{10}H_{21}O_8P$. Calculated. C 40.00, H 7.00, OCH_3 51.6, P 10.32
Found. " 40.35, " 7.19, " 51.81, " 10.37

Dephosphorylation Experiments

Two 0.1 gm. samples of the fully methylated phosphoribose were dissolved in 25 cc. of water containing respectively 2 molecular equivalents of hydrochloric acid and sodium hydroxide. Small portions were sealed in test-tubes and heated in an oil bath at 140°. At stated intervals the amount of free phosphorus was determined by the Kuttner-Cohen[3] method. After 2½ hours the acid solution was found to be 99 per cent hydrolyzed, while the basic solution liberated only 12 per cent of its phosphorus in 16 hours. Only 50 per cent of free sugar was found by the Hagedorn-Jensen[4] titration. At steam bath temperature the rate of hydrolysis with 0.1 N HCl was very much slower. Hydrolysis in ammonium acetate solution at 140° showed less than 5 per cent free phosphorus. An attempted hydrolysis of 2 gm. of material in a sealed tube at 140–150° showed only 70 per cent liberation of free phosphorus.

Oxidation of the New Ribosephosphoric Acid by Means of Nitric Acid—The procedure was essentially that of Levene and Tipson.[5] After the removal of the nitric acid by distillation, the residue was transferred to a distilling flask with acetone. The acetone was replaced with methyl alcohol. After evaporation to a syrup, it was dissolved in methyl alcohol containing 2 per cent of hydrogen chloride. This solution was refluxed for 1 hour. The methyl alcohol was removed by distillation and the distillate was boiled with an excess of barium hydroxide. A precipitate was formed which was proved, by qualitative tests, to be barium oxalate. The residue left after the distillation of the methyl alcohol was thoroughly extracted with ether. No appreciable amount of material was found in the ether extract. The methyl ester of dimethyltartaric acid would have been found here if any had been formed.

---

[3] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927). See also Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **79**, 621 (1928).

[4] Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923). See also Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

[5] Levene, P. A., and Tipson, R. S., *J. Chem. Soc.*, **93**, 623 (1931).

The ether-insoluble residues contained an acid or acids with a very low phosphorus content. The only positive result from this experiment was the isolation of oxalic acid as the barium salt.

Reduction with Sodium Amalgam—The experiment was first tried with barium hexose-3-phosphate. Catalytic reduction proceeded very slowly and so reduction with sodium amalgam was attempted. The Fehling's test was practically negative after 50 to 60 hours and the Hagedorn-Jensen titration showed 97 per cent reduction. Very little phosphorus was liberated in this process. The same method was tried with barium ribosephosphate. Only 2 gm. of product were isolated from 10 gm. of starting material. The material was not very pure as shown by its analysis. The substance exhibited a slight optical activity.

Catalytic Reduction—5 gm. of barium ribosephosphate were dissolved in 25 cc. of water to which 0.5 gm. of platinum oxide catalyst was added. This mixture was shaken with hydrogen at 40 pounds pressure for 20 hours.

A Hagedorn-Jensen titration showed that 98 per cent reduction had taken place. The catalyst was filtered off, and the solution diluted, filtered, and concentrated to a volume of 10 to 15 cc. The salt was precipitated by pouring into absolute alcohol. It was purified by dissolving in a small amount of water, making slightly alkaline with barium hydroxide, and reprecipitating with absolute alcohol. Analysis showed that it had the constitution of barium ribitolphosphate.

4.500 mg. substance: 2.660 mg. CO_2 and 1.300 mg. H_2O
4.620 " " 26.20 " ammonium phosphomolybdate
0.1000 gm. " 0.0620 gm. $BaSO_4$
$C_5H_{11}O_8PBa$. Calculated. C 16.33, H 2.99, P 8.44, Ba 37.40
Found. " 16.11, " 3.23, " 8.23, " 36.49

The rotation was taken in the following manner. Exactly 0.5 gm. of barium ribitolphosphate was dissolved in 1 to 2 cc. of water and treated with an exact equivalent of sodium oxalate. The barium oxalate was filtered, washed, and the filtrate evaporated to a small volume. This was filtered, diluted to 10 cc., and the optical rotation taken in a 2 dm. tube. The reading was 0°. 5 cc. of this solution were diluted with 5 cc. of saturated borax solution (25°) and the optical rotation was again taken. The reading was less than +0.01° (±0.02°).

The same procedure with the same quantities was carried out with barium ribosephosphate. The rotation calculated on the basis of sodium ribosephosphate is given below.

$$[\alpha]_D^{26} = \frac{-0.67^\circ \times 100}{3.749 \times 2} = -8.93^\circ \text{ (in water)}$$

When 5 cc. of this solution were diluted with 5 cc. of borax solution, the reading was

$$[\alpha]_D^{26} = \frac{+1.53^\circ \times 100}{\frac{3.749}{2} \times 2} = +40.81^\circ$$

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, October, 1932,
Vol. 98, No. 1, pp. 17–19]

A NOTE ON THE USE OF 1-BROMOTETRAMETHYLGLUCOSE FOR THE SYNTHESIS OF METHYLATED GLYCOSIDES

BY P. A. LEVENE AND FRANK CORTESE

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, July 12, 1932)

When synthesis is undertaken for the purpose of establishing the structure of a substance, the task is accomplished equally well if the synthesis of a known derivative of the substance is attained. Inasmuch as modern methods have made it feasible to methylate practically all glycosides, it is sufficient for structural purposes to be able to synthesize the methylated glycosides. The advantages which these offer are many and one of the most important is that they are volatilizable. Thus far only few similar syntheses are recorded; namely, those of Freudenberg and his collaborators[1] who employed for this purpose 1-chlorotetramethylglucose and 1-chloroheptamethylcellobiose. We thought that 1-bromotetramethylglucose which is more accessible than the chloro derivative might serve the purpose to advantage.

In this note the method of preparation of 1-bromotetramethylglucose is reported and in a preliminary way its conversion into tetramethylmethylglucopyranoside and into theophylline-tetramethylglucopyranoside. The details of preparation are given in the experimental part. Still better results than those reported undoubtedly will be obtained when larger quantities of material will be used so as to permit further purification.

EXPERIMENTAL

Acetylation of Tetramethylglucose—40 gm. of tetramethylglucose were dissolved in 200 cc. of acetic anhydride. 15 gm. of freshly fused sodium acetate were added and the mixture was refluxed 10 minutes. After cooling, 200 cc. of dry ether and 300 cc. of toluene were added and the whole was evaporated to a syrup under diminished pressure in a bath at 50°. This evaporation was repeated twice with two portions of toluene (200 cc.). The product was dissolved in dry ether, the sodium acetate filtered off, and the filtrate evaporated to a syrup which was distilled with an oil pump. The fraction boiling at 133–138° was collected. The yield of thick colorless and odorless oil amounted to 37.3 gm.

---

[1] Freudenberg, K., Andersen, C. C., Go, Y., Friederich, K., and Richtmyer, N. W., *Ber. chem. Ges.*, **63**, 1964 (1930).

The substance had the following composition.

3.665 mg. substance: 7.00 mg. CO_2 and 2.570 mg. H_2O
3.291 " " : 10.290 " AgI
$C_{12}H_{22}O_7$. Calculated. C 51.80, H 7.91, OCH_3 44.60
Found. " 52.08, " 7.84, " 41.27
0.1644 gm. substance required 5.91 cc. 0.1 N NaOH
$C_{12}H_{22}O_7$. Calculated, CH_3CO 15.46; found, CH_3CO 15.46

1-Bromo-2,3,4,6-Tetramethylglucose—10 gm. of tetramethylglucose acetate were dissolved in a solution of 60 gm. of hydrogen bromide in 60 gm. of glacial acetic acid. The reaction mixture was kept in a bath of ice water for 1 hour, after which the solution was diluted with large amounts of dry ether and toluene and concentrated to a syrup under diminished pressure in a bath at 50°. This evaporation was repeated twice with two 200 cc. portions of toluene. The residual syrup amounted to 10 gm. and was initially light yellow but soon changed to a deep red. In fact, the compound is very unstable.

The substance had the following composition.

0.1120 gm. required 3.38 cc. 0.1 N $AgNO_3$
$C_{10}H_{19}O_5Br$. Calculated, Br 26.85; found, Br 24.32

Methylation of 1-Bromo-2,3,4,6-Tetramethylglucose—10 gm. of freshly prepared bromotetramethylglucose were treated with 50 cc. of dry methyl alcohol and 12 gm. of dry silver carbonate. Charcoal was added and the mixture was filtered. After evaporating the alcohol under diminished pressure, the residual syrup was distilled with an oil pump. 4 cc. distilled over and about 3 cc. remained as a deep red viscid tar. The distillate was fractionated at high vacuum and was shown to be one-third tetramethyl- and two-thirds pentamethylglucose.

The substances had the following composition.

Fraction I

4.686 mg. substance: 9.094 mg. CO_2 and 3.610 mg. H_2O
3.865 " " : 17.06 " AgI
$C_{11}H_{22}O_6$. Calculated. C 52.80, H 8.80, OCH_3 62.00
Found. " 52.92, " 8.62, " 59.04

Fraction II

3.988 mg. substance: 7.530 mg. CO_2 and 2.960 mg. H_2O
3.753 " " : 14.152 " AgI
$C_{10}H_{20}O_6$. Calculated. C 50.85, H 8.47, OCH_3 52.5
Found. " 51.49, " 8.30, " 49.8

Theophylline-2,3,4,6-Tetramethylglucoside—15 gm. of dry silver theophylline were added to a solution of 10 gm. of 1-bromo-2,3,4,6-tetramethylglucose in 190 cc. of dry xylene and the mixture boiled for 45 minutes under a reflux. After cooling, the solution was filtered into 1500 cc. of petroleum ether. This was then filtered and the liquid concentrated under diminished pressure. The residue, consisting of 6 cc. of a yellow viscid gum, was distilled at 0.006 mm. to give the following fractions.

Bath	Fraction No.	Amount	Description
°*C.*		*cc.*	
120–140	I	1.6	Colorless and mobile
140–180	II	1.0	Very viscous, deep yellow gum
180–220	III	2.0	Solidified on cooling into deep yellow transparent solid
	Residue	1.0	Deep red transparent solid

Fraction III—This was redistilled and had the following composition.

3.676 mg. substance: 0.406 mg. N_2 (25° and 738 mm.)
$C_{17}H_{26}O_7N_4$. Calculated, N 13.22; found, N 12.28

The glucoside did not reduce Fehling's solution until after acid hydrolysis.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, October, 1932, Vol. 98, No. 1, pp. 109–114]

SERINEPHOSPHORIC ACID OBTAINED ON HYDROLYSIS OF VITELLINIC ACID

BY FRITZ A. LIPMANN* AND P. A. LEVENE

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, July 20, 1932)

The discovery in nature of many new organic compounds containing a phosphoric acid residue and the realization of their biological significance have caused renewed interest in the natural conjugated phosphoric acids that have been known for a long time but whose structure had not been known. Among the latter are the phosphoproteins. The best known representatives of these are casein and vitellin. From the latter Levene and Alsberg[1] obtained by treatment with ammonia water (12 per cent) for 2 hours a substance of constant composition containing 10 per cent of phosphorus and about 14 per cent of nitrogen, having the ratio P:N = 1:3. 26 years later Posternak and Posternak[2] prepared a substance of similar composition by tryptic digestion of vitellin. Undoubtedly these authors were dealing with the vitellinic acid of Levene and Alsberg. Posternak and Posternak subjected the substance to hydrolysis and found among the products of hydrolysis a higher proportion of serine than had hitherto been found in any other protein. The high yield of ammonia on acid hydrolysis and the isolation of pyruvic acid on alkaline hydrolysis, according to these authors, also was indicative of a high proportion of serine.[3] On the basis of the experience of Bergmann and his coworkers,[4] such results should be expected from a serine peptide. Later, the authors prepared a substance with a ratio of P:N = 1:1.3 and even 1:1. Analytical data on these substances were not presented.

---

* Fellow of the Rockefeller Foundation.

[1] Levene, P. A., and Alsberg, C. L., *J. Biol. Chem.*, **2**, 127 (1906–07).

[2] Posternak, S., and Posternak, T., *Compt. rend. Acad.*, **184**, 909 (1927).

[3] Posternak, S., and Posternak, T., *Compt. rend. Acad.*, **187**, 313 (1928).

[4] Bergmann, M., Miekeley, A., Weinmann, S., and Kann, E., *Z. physiol. Chem.*, **143**, 108 (1925). Bergmann, M., Miekeley, A., and Kann, E., *Z. physiol. Chem.*, **146**, 247 (1925).

Contemporaneously with Posternak and Posternak, Rimington[5] was conducting investigations on casein peptone and prepared a substance with 7 per cent phosphorus and 10 per cent nitrogen, the ratio of P:N being 1:3. He reached the conclusion that the phosphoric acid residue in his peptone was bound to hydroxyglutamic and hydroxybutyric acids. His conclusion, however, was disputed by Posternak[6] who insisted that the phosphoric acid residue was bound to serine in the substance from casein as well.

The present investigation was undertaken with a view to a more detailed study of the structure of vitellinic acid than was presented by Posternak and Posternak. The contributions of these authors are undoubtedly of great importance but many of their results are of a qualitative character.

The vitellinic acid was prepared by the procedure of Levene and Alsberg with the sole modification that for purification it was precipitated from an aqueous solution by means of hydrochloric acid and thus all impurities were removed by washing with water. The composition was practically the same as previously found by Levene and Alsberg. The important point concerning the peptide part of the substance is its high nitrogen content, namely 17 per cent, which indicates a high proportion of the basic amino acids. Indeed, a rough estimation by the phosphotungstic acid method showed that 40 per cent of the total nitrogen belonged to the basic amino acids. On the basis of this consideration, it must be concluded that of the total nitrogen of the peptide, there was only 70 per cent in the form of amino groups. Hence, in the vitellinic acid there should be present one phosphoric acid residue to every two amino acids. Furthermore, if there is in the molecule only one definite amino acid to which the phosphoric acid is attached, then it should be possible to isolate it without much difficulty.

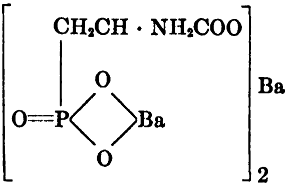
On dephosphorylation with 0.25 N alkali the polypeptide is obtained in practically unchanged condition. It, similarly to the peptide part of vitellinic acid, contains 17 per cent nitrogen, and, similarly to the original vitellinic acid, yields on hydrolysis with mineral acid about 18 per cent ammonia. Of this not more than 25 per cent originates in amide nitrogen. This finding is in disagreement with the conclusion of Posternak and Posternak who thought the high ammonia formation is conditioned by the presence of the phosphoric acid residue. This high yield of ammonia may be regarded as an indication of the presence of a high proportion of serine in the molecule of vitellinic acid.

---

[5] Rimington, C., *Biochem. J.*, **21**, 272, 1179 (1927).

[6] Posternak, S., *Compt. rend. Acad.*, **186**, 1762 (1928).

The most important fact to be reported in this communication is the isolation from the product of partial hydrolysis of vitellinic acid of a barium salt which had the composition of the salt of serinephosphoric acid.



The details of the preparation are given in the experimental part.

EXPERIMENTAL

Preparation of Vitellinic Acid—The procedure of Levene and Alsberg was followed in the main features. The ammoniacal solution was treated with picric acid and acetic acid was added to about pH 5 (calculated so that the proportion of ammonium acetate to acetic acid was 3:1 to 2:1). The crude vitellinic acid was precipitated with a minimum volume of alcohol. The crude substance was redissolved in water and reprecipitated with hydrochloric acid. The precipitate was washed free of picric acid and of ammonia and then dried by treatment with alcohol of progressively increasing concentration. The substance was practically insoluble in cold water, very slightly soluble in hot water, and the solution was acid to Congo red. On titration with thymolphthalein a solution of the substance consumes 2 equivalents of sodium hydroxide for each phosphorus atom, showing that in the region of pH 8, there are no titratable acidic groups in the substance and that only 1 equivalent of phosphoric acid is bound to the substance.

The proportions of nitrogen to phosphorus were as follows:

	N	P	N:P
First preparation..............	10.19	12.71	2.76
Second "	10.45	12.7	2.69
Third "	10.12	12.33	2.70

These values show a fairly constant composition and particularly a very constant relationship of nitrogen to phosphorus.

Dephosphorylation of Vitellinic Acid and the Dephosphorylated Polypeptide

It is known that dephosphorylation of all phosphoproteins proceeds very rapidly on the alkaline side and but very slowly in dilute acid. Thus, at pH 11.1 at 100° the substance loses 80 per cent of its phosphoric acid in 3½ hours, whereas in the presence of 2.0 N hydrochloric acid at the same temperature the loss in 10 hours is only 38 per cent. For the preparation of the polypeptide free from phosphoric acid, vitellinic acid was dissolved in a solution of 0.25 N sodium hydroxide and allowed to stand for 60 hours at 31°. To the solution ammonium acetate and magnesia mixture are added as long as a precipitate is formed. The filtrate from this precipitate is acidulated to pH 5, when the polypeptide settles out. To complete precipitation an equal volume of methyl alcohol is added. The precipitate is washed carefully with dilute methyl alcohol, faintly acidulated with acetic acid, and finally dried with methyl alcohol of progressively increasing concentration.

The nitrogen content of several samples of protein prepared in this manner is 17.48, 17.53, 17.56, and 17.28 per cent. The nitrogen content of the polypeptide of the vitellinic acid calculated from the nitrogen content of vitellinic acid likewise is 17 per cent, thus showing that on dephosphorylation the protein undergoes little change. The yield of the polypeptide was 70 per cent of the theory.

Ammonia Formation on Acid Hydrolysis of Vitellinic Acid and of Its Protein Component

For the purpose of comparing the ammonia formation of the two substances, each was hydrolyzed for 36 hours with 20 per cent hydrochloric acid. The ammonia estimation was made by the method of Parnas.[7] The values for the vitellinic acid were found to be 17.3 per cent and for the dephosphorylated substance 17.9 per cent.

In order to determine the origin of the ammonia, the vitellinic acid was digested with 0.25 N sodium hydroxide solution for 60 hours at 31°. The yield of ammonia was 4.6 per cent of the total nitrogen. In order to test whether the treatment was sufficiently vigorous to hydrolyze all the amide nitrogen, asparagine was submitted to the same treatment, with a yield of 84 per cent nitrogen of the nitrogen present in the substance in the form of amide nitrogen. Thus, it is warranted to assume that not more than one-third of the ammonia developed on acid hydrolysis originated in the amide nitrogen of the polypeptide.

Isolation of the Serinephosphoric Acid—Vitellinic acid is hydrolyzed for 10 hours with 2.0 N hydrochloric acid. 38 per cent of the phosphoric acid is cleaved off. The solution is neutralized with barium carbonate and then made alkaline to phenolphthalein. About one-third of the bound phosphoric acid remains in solution and two-thirds, together with the inorganic phosphate, in the precipitate. This precipitate is dissolved in acetic acid and the solutions neutralized to litmus with ammonia water. A precipitate of inorganic phosphate with a small amount of organic phosphate is filtered off and the filtrate combined with the first filtrate is made alkaline to phenolphthalein by means of a solution of barium hydroxide. The substance is precipitated from the solution by means of 50 per cent alcohol. The precipitate is nearly completely soluble in a considerable volume of water. The aqueous solution is precipitated with 50 per cent alcohol. The ratio of N:P in this crude substance was 1.3:1; the ratio of NH_2 (Van Slyke):P is 1:1. The yield was 60 per cent of the phosphorus not cleaved off by hydrochloric acid.

For further purification the substance was redissolved in 2.0 N hydrochloric acid (an insoluble precipitate settled out which was removed) and the solution was allowed to digest an additional 3 hours. The solution was then neutralized to litmus, filtered from the greater part of the inorganic phosphates, and the filtrate was made alkaline with a solution of barium hydroxide. To the solution alcohol is added to 50 per cent concentration. To remove all of the inorganic phosphate the precipitate is extracted with a large volume of water. To the aqueous solution containing nearly all the organic phosphate, alcohol is added to 25 per cent concentration. One part of the salt remains in solution, which, when precipitated with more alcohol has the composition of the dibasic salt of serinephosphoric acid.

---

[7] Parnas, J. K., and Klisiecki, A., *Biochem. Z.*, **173**, 227 (1926).

5.400 mg. substance: 33.270 mg. P_2O_7
8.950 " " : 0.353 cc. N at 29° and 753 mm.
0.0648 gm. " : 0.0453 gm. $BaSO_4$
4.785 mg. " : 1.915 mg. CO_2 and 0.930 mg. H_2O
$C_3H_6NO_6PBa$. Calculated. C 11.2, H 1.82, N 4.36, P 9.65, Ba 42.7
Found. " 10.9, " 2.18, " 4.4, " 8.95, " 41.0

The material obtained by precipitation with 25 per cent alcohol was taken up in water. The greater part dissolved. Alcohol was then added to 50 per cent concentration and to the suspension a very dilute solution of barium hydroxide was added drop by drop to alkaline reaction to thymolphthalein. The precipitate was rapidly centrifuged and the centrifuge washed with 50 per cent alcohol, 90 per cent alcohol, and ether. Washing with alcohol of greater strength is not advisable. This substance had the composition of the tribasic salt of serinephosphoric acid. All of the nitrogen is present as NH_2 nitrogen (Van Slyke).

$(C_3H_5NO_6PBa)_2Ba$. Calculated. C 9.36, H 1.50, N 3.42, P 8.06, Ba 52.38
Found. " 9.27, " 1.30, " 3.60, " 7.99, " 53.09

The optical rotation of the barium salt ($c = 1.70$) dissolved in 10 per cent hydrochloric acid was $[\alpha]_D^{25} = +0.14°$. The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+0.14° \times 100}{1 \times 1.70} = +8.2° \ (\pm\ 0.02°)$$

Calculated as the free acid, the rotation was

$$[\alpha]_D^{25} = \frac{+0.14° \times 100}{1 \times 0.81} = +\ 17.3°$$

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, November, 1932, Vol. 98, No. 2, pp. 735–738]

PHYTOCHEMICAL REDUCTION OF 1-HYDROXY-2-OXO-HEPTANE (HEPTANOL-1-ONE-2)

BY P. A. LEVENE AND A. WALTI

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, September 16, 1932)

In the work on the correlation of the configurations of optically active α-hydroxy acids and of secondary carbinols, the corresponding glycols were frequently made use of. When the glycols were needed as reference substances, they were prepared by the reduction of the corresponding α-hydroxy aldehydes, which, in their turn, had been prepared by the ozonization of the unsaturated secondary carbinols. When larger quantities of the optically active glycols were required for the purpose of the preparation of secondary carbinols, the phytochemical reduction of the corresponding α-hydroxy ketones was resorted to. In connection with the work on the configurational relationship of α-hydroxyheptanoic acid to other α-hydroxy acids, the optically active heptanediol-1,2 was prepared by both methods. The one obtained by the reduction of the aldehyde has been previously described.[1] The phytochemical method is described in the present publication inasmuch as the procedure used in the case of the lower α-hydroxy ketones had to be modified in some details for the higher homologues because of their lower solubility in water.

EXPERIMENTAL

dl-1-Chloro-2-Hydroxyheptane—This substance was obtained on condensation of equivalents of chloroacetaldehyde and amyl magnesium bromide. The chloroacetaldehyde was freshly prepared from 450 gm. (3 mols) of chloroacetal by slowly distilling it over 300 gm. of anhydrous oxalic acid. The distillate was taken up in dry ether and shaken for 3 hours with pulverized calcium chloride, filtered, and the filtrate dropped into a well cooled ether solution of 3 mols of amyl magnesium bromide. After all the chloroacetaldehyde had been added, the reaction mixture was immediately decomposed by pouring it onto ice and acidifying with hydrochloric acid. The ethereal part was removed and the aqueous part twice extracted with liberal quantities of ether. The united ether solution was washed successively with water, sodium carbonate, and water until neutral, and dried overnight over sodium sulfate. After refractionation the chlorohydrin distilled at 93° at 13 mm. and had the following composition.

---

[1] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **94**, 593 (1931).

0.1244 gm. substance: 0.1194 gm. AgCl
$C_7H_{15}OCl$ (150.61). Calculated. Cl 23.54
Found. " 23.74

The yield varied from 30 to 45 per cent of the calculated amount.

1-Chloro-2-Oxo-Heptane (*1-Chloroheptanone-2*)—The oxidation of the chlorohydrin to the chloroketone was carried out in the following manner: 100 gm. of the heptylene chlorohydrin and 65 gm. of pulverized sodium dichromate were cooled with ice and water and mechanically stirred, and 158 cc. of a 50 per cent sulfuric acid solution added in such a manner that the temperature was kept between 10–20°. When all the acid had been added, the mixture was stirred 1 hour more at room temperature and then was diluted with water and extracted with ether. The washed and dried solution (over sodium sulfate) was fractionated. The chloroketone distilled at 80° at 13 mm. The substance had the following composition

4.125 mg. substance: 8.545 mg. CO_2 and 3.240 mg. H_2O
$C_7H_{13}OCl$ (148.59). Calculated. C 56.55, H 8.82
Found. " 56.49, " 8.78

The yield was about 90 per cent of the calculated amount.

1-Hydroxy-2-Oxo-Heptane (*Heptanol-1-One-2*)—This hydroxy ketone was readily obtained by warming on a steam bath the chloroketone just described with its own weight of methanol and 1.25 mols of anhydrous potassium formate until the irritant odor of the chloroketone had disappeared (overnight).

After cooling, anhydrous ether was added and the precipitated salts were removed by filtration on a Buchner funnel. The salt was extracted with ether. On concentration of the ethereal solution a fresh precipitate of salts formed and was removed. The final concentrate was fractionated, the hydroxyheptanone distilling at 87° at 13 mm. On redistillation it distilled at 95° at 20 mm. The substance crystallized in the receiver, the cooling water having a temperature of 10°. It reduced Fehling's solution in the cold. The substance had the following composition.

4.960 mg. substance: 11.660 mg. CO_2 and 4.795 mg. H_2O
$C_7H_{16}O_2$ (130.14). Calculated. C 64.57, H 10.84
Found. " 64.10, " 10.82

Dextro-Heptanediol-1, 2—To an actively fermenting mixture of 1 kilo of sugar and 1 kilo of bakers' yeast and 10 liters of tap water was added drop by drop a solution of 100 gm. of heptanol-1-one-2 in 150 cc. of alcohol. *The mixture was stirred continuously.* On each of 5 successive days, about 100 gm. of fresh yeast were added. After 6 days the mixture was worked up. If a filtered sample still reduced Fehling's solution, the material was extracted with ether to recover most of the unchanged hydroxy ketone for further fermentation. The aqueous part was concentrated to a viscous liquid under reduced pressure. It was then treated with absolute alcohol and a small amount of dry ether. The precipitate was removed by filtration on a Buchner funnel and washed with alcohol-ether. The filtrates were concentrated again and the residue once more treated with absolute alcohol and an equal amount of dry ether. The filtrate was concentrated and fractionated. The main fraction of the heptylene glycol distilled at 97–98° at 1.5 mm. or at 93–94° at 1 mm. The substance had the rotation of $\alpha_D^{25} = +2.9°$. On redistillation the middle fraction (b.p. 121–122° at 12 mm.) had a rotation of $\alpha_D^{22} = +3.30°$. The specific rotation of the substance was

$$[\alpha]_D^{22} = \frac{+1.97° \times 100}{1 \times 11.72} = +16.81° \text{ (in absolute alcohol)}$$

The composition of the substance was as follows:

4.491 mg. substance: 10.460 mg. CO_2 and 4.780 mg. H_2O
$C_7H_{16}O_2$ (132.16). Calculated. C 63.59, H 12.20
Found. " 63.51, " 11.91

Diphenylurethane of Dextro-Heptanediol-1,2—The diphenylurethane of this glycol was prepared in the usual manner. It was twice recrystallized from dilute alcohol. It melted at 111–112° and had the following composition.

0.1000 gm. substance: 5.63 cc. 0.1 N HCl (Kjeldahl)

$C_{21}H_{26}O_4N_2$ (370.32).	Calculated.	N 7.57
	Found.	" 7.88

The urethane had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+1.45° \times 100}{1 \times 11.94} = +12.14° \text{ (in absolute alcohol)}$$

Dextro-1-Bromo-2-Hydroxyheptane—This active heptylene bromohydrin was prepared in a manner similar to that described for the levo-propylene bromohydrin from levo-propylene glycol.[2] 24 gm. of dry hydrogen bromide were passed into 35 gm. of dextro-heptylene glycol (α_D = +2.85°) which was kept in an ice-water mixture. The reaction mixture was allowed to stand overnight. Chloroform and ice were then added and the product worked up as mentioned for levo-propylene bromohydrin. On distillation a main fraction (30 gm.) was obtained which distilled at 102° at 14 mm. The second fraction (10 gm.) distilled at 103–117° at 14 mm. and was less pure. On redistillation the first fraction distilled at 75° at 1.5 mm. Its composition was as follows:

0.1307 gm. substance: 0.1236 gm. AgBr

$C_7H_{15}OBr$ (195.07).	Calculated.	Br 40.97
	Found.	" 40.24

The rotation of the substance was α_D^{22} = +2.0° (without solvent).

[2] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **68**, 415 (1926).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, September, 1932, Vol. 97, No. 3, pp. 727-737]

STROPHANTHIN

XXVII. RING III OF STROPHANTHIDIN AND RELATED AGLUCONES

BY WALTER A. JACOBS AND ROBERT C. ELDERFIELD

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 21, 1932)

In previous work two of the rings of strophanthidin, and therefore of related aglucones, have been shown to be 6-membered.[1] More recently, new observations have been made which furnish evidence in regard to a third ring. On another occasion a monoanhydrodihydrostrophanthidin[2] was described, which was obtained by the action of acid on dihydrostrophanthidin. This substance has now become the important starting material for the series of substances which have given evidence as to the position and size of Ring III. It is formed by the loss of the tertiary hydroxyl group, OH^{I}, of dihydrostrophanthidin as water. Since dihydrostrophanthidin has the partial structure given in Formula I, the double bond of the monoanhydro derivative may have one of three positions (Formulæ II, III, and IV).

On oxidation of this substance in acetone solution with permanganate an acid, *monoanhydrodihydrostrophanthidinic acid*, of the formula $C_{23}H_{32}O_6$, was obtained, which resulted from the oxidation of the aldehyde group to carboxyl. If, however, the oxidation was performed in alkaline solution, a different acid was formed of the formula $C_{23}H_{34}O_8$, which resulted from the oxidation of the aldehyde group to carboxyl and the addition of two hydroxyl groups to the double bond. This acid readily formed a *methyl ester* which on acylation formed a *di-p-bromobenzoate*. On the basis of the locations assigned to the double bond in Formulæ II, III, and IV the new hydroxyl groups may have the positions given in Formulæ V, VI, and VII. When the ester was oxidized with chromic acid, an acid was formed which on analysis proved to have the formula $C_{24}H_{32}O_9$. Titration showed that the substance is a monobasic acid and it readily neutralizes a second equivalent of alkali on saponification, due to the original lactone group still present. Only a fraction of a third equivalent was consumed under the conditions employed, due to the partial saponification of the resistant methyl ester group. Further work showed that this acid was produced by the rupture of a ring between a secondary and a tertiary hydroxyl group with the formation of a carboxyl and a carbonyl group. Formulæ II and V are therefore excluded from further consideration.

---

[1] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 74, 805 (1927); 92, 323 (1931).

[2] Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 74, 791 (1927).

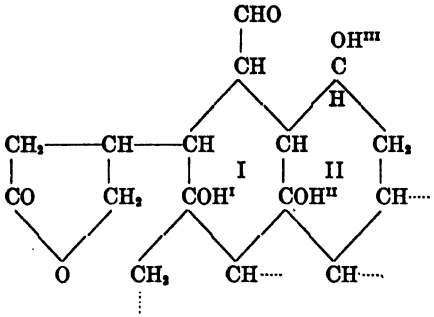
Simultaneously, the original secondary hydroxyl group of dihydrostrophanthidin (OH^{III}) was also oxidized to the carbonyl. The acid is therefore a monomethyl ester of a dibasic diketo hydroxylactone acid, the *dimethyl ester* of which was readily prepared with diazomethane. For the former, any one of Formulæ VIII, IX, and X might be considered. In order to make a decision from among these, it was hoped to show the retention in this substance of the original OH^{II} of dihydrostrophanthidin by its behavior towards acids. If present, OH^{II} would be β to the keto group formed from OH^{III} and therefore form an unsaturated anhydro ketone, as in the case of the formation of anhydroisostrophanthonic acid from isostrophanthonic acid. This would at once eliminate Formula X. Both the acid and the ester were easily altered by acids but the reaction products were unworkable resins. In attempts also to prepare carbonyl derivatives no crystalline reaction products could be isolated.

The desired evidence was furnished by hydrogenation. Catalytic hydrogenation of the diketo acid was accomplished in ethyl acetate solution with the catalyst of Adams and Shriner. The reaction proceeded very slowly. The main product was neutral and on analysis gave figures in agreement with the formula $C_{24}H_{34}O_8$. A relatively small acid fraction was also formed. On attempting, however, to isolate the free acid, it was found to lactonize readily with the formation of a neutral substance which is apparently isomeric with the main neutral reaction product. In the formation of the latter it was obvious that the two carbonyl groups had been reduced to hydroxyl groups on one of which the carboxyl group lactonized with loss of water. It gave only a *monoacetate.* The substance must be a *dihydroxydilactone ester.* This was confirmed by its behavior towards alkali. The ester group still exhibited a relative resistance towards alkali. On reacidification, the two saponified lactone groups readily closed again with the reformation of the dilactone ester. This behavior at once restricts the relative position of the carboxyl and carbonyl groups from which the new lactone group is formed to γ or δ. Formula X is, therefore, eliminated and the dilactone ester can have only Formula XI or XII.

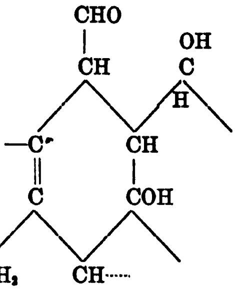
The decision between these possibilities depended upon the determination of the identities of the groups involved in the new lactone group. This was established as follows: On reoxidation, a *hydroxyketodilactone ester,* $C_{24}H_{32}O_8$ (Formula XIII), was formed, which was further characterized by its *oxime.* The probability that the carbonyl group was formed from OH^{III} and that OH^{II} of the original strophanthidin molecule persisted through the whole series of reactions was supported by the ready formation of an *anhydroketodilactone ester,* $C_{24}H_{30}O_7$ (Formula XIV), by the action of dilute acid. The above dihydroxydilactone ester is stable under the same conditions. Formula XI, and not XII, therefore, represents this dihydroxydilactone ester. The formation of a *neutral sulfite* (Formula XV) by the action of thionyl chloride is a confirmation of this conclusion.[3]

It appears most likely that the new lactone group is δ and not γ. The former has its origin in the cleavage of a pentacyclic and the latter in that of a less probable tetracyclic system. We shall, therefore, provisionally accept from the present evidence that Ring III of strophanthidin and related aglucones is pentacyclic. Its position as given is apparently definitely established. It is joined to Ring I at carbon atoms (5) and (6) (Formula XVI). Whether it is substituted or not is a conclusion which must await more data. From certain reactions there is a suggestion that a methyl group is attached to Ring III on carbon atom number (17). It is not impossible that Ring IV will be either 5- or 6-membered and joined to carbon atoms (14) and (15) of Ring II. A provisional incompleted formula for strophanthidin might be represented as in Formula XVI.

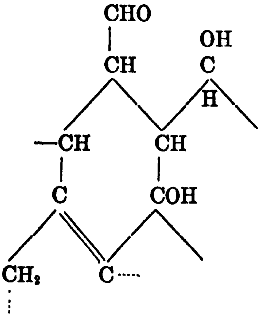
[3] Jacobs, W. A., Elderfield, R. C., Grave, T. B., and Wignall, E. W., *J. Biol. Chem.*, **91**, 623 (1931).



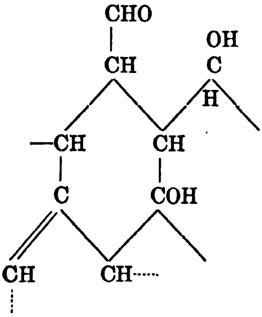
I



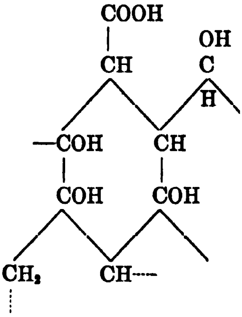
II



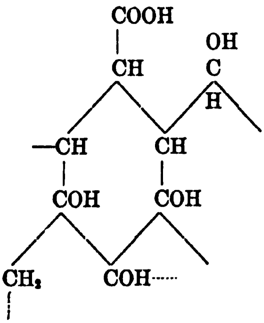
III



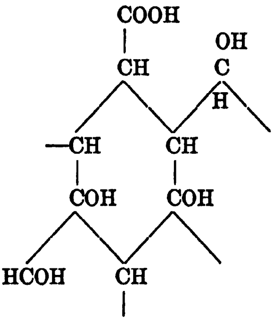
IV



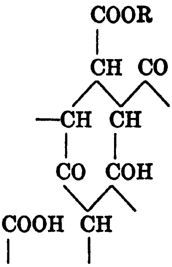
V



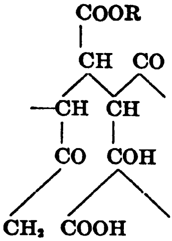
VI



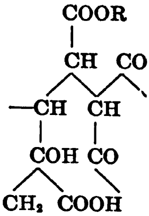
VII



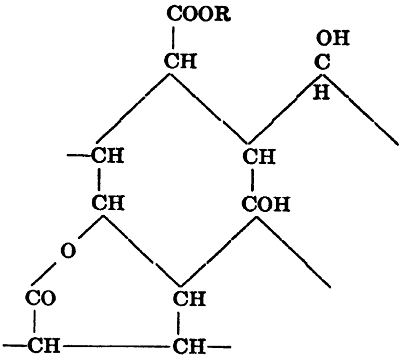
VIII



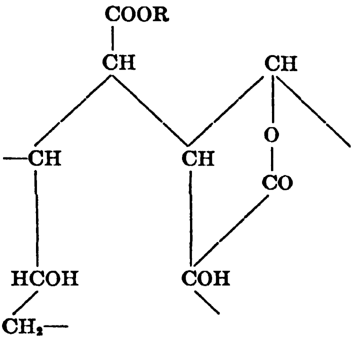
IX



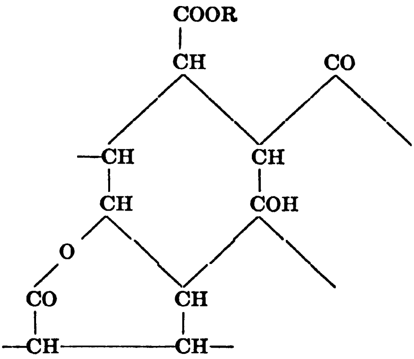
X



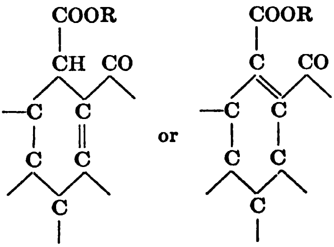
XI



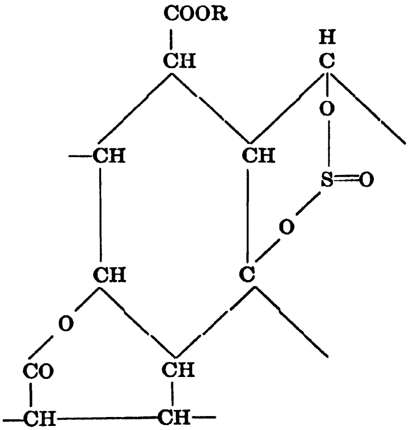
XII



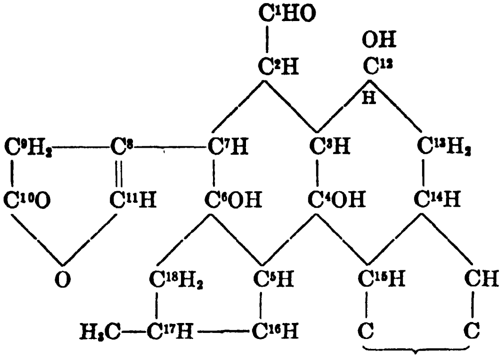
XIII



XIV



XV



XVI

EXPERIMENTAL

Monoanhydrodihydrostrophanthidinic Acid, $C_{23}H_{32}O_6$—1 gm. of monoanhydrodihydrostrophanthidin was dissolved in 100 cc. of dry acetone. 0.9 gm. of finely powdered $KMnO_4$ was added and the mixture was stirred mechanically. Oxidation occurred promptly. After 2 hours the MnO_2 was filtered off and thoroughly extracted with water. The aqueous extract was acidified to Congo red with HCl, and the acid which precipitated was collected. Upon recrystallization by careful dilution of its acetone solution it formed irregular platelets and melted at 185–186° with decomposition. The substance was soluble in dilute Na_2CO_3 solution.

$[\alpha]_D^{24} = +72°$ (c = 0.800 in pyridine)
4.847 mg. substance: 3.355 mg. H_2O, 12.045 mg. CO_2

$C_{23}H_{32}O_6$.	Calculated.	C 68.27, H 7.98
	Found.	" 67.77, " 7.74

The same substance was also obtained, although in poorer yield, if the oxidation was carried out in dry pyridine.

Tetrahydroxylactone Acid, $C_{23}H_{34}O_8$ (*Formula VII*)—20 gm. of monoanhydrodihydrostrophanthidin were dissolved in 150 cc. of pyridine and 800 cc. of 0.1 N NaOH were slowly added while the mixture was stirred mechanically. After 30 minutes, saponification of the lactone was complete. If the alkali is added at once, reprecipitation occurs and a longer time is required for resolution. 350 cc. of 5 per cent $KMnO_4$ solution were then added during 2 hours, with the temperature kept below 20°. The filtrate from the MnO_2 was acidified to litmus with acetic acid and then concentrated in a vacuum to 300 cc. It was then acidified to Congo red with HCl. On standing, the acid deposited as long, silky needles. The yield of crude acid was 11 gm. After recrystallization from dilute acetone, it melted at 215–218° with decomposition after preliminary softening at about 160°. The melting point varied considerably.

4.122 mg. substance: 2.915 mg. H_2O, 9.555 mg. CO_2
$C_{23}H_{34}O_8$. Calculated. C 62.98, H 7.82
Found. " 63.22, " 7.91

13.840 mg. of substance were titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent, 0.317 cc.; found, 0.306 cc. 3 cc. of 0.1 N NaOH were then added and the mixture was refluxed for 4 hours and then titrated back against phenolphthalein. 0.350 cc. of additional 0.1 N NaOH were consumed.

Methyl Ester of Tetrahydroxylactone Acid—The above acid was esterified in acetone solution with diazomethane. The ester is very readily soluble in all the usual organic solvents except petrolic ether. It crystallizes from ether as aggregates of platelets which melt at 207–208°.

$[\alpha]_D^{28} = +82°$ (c = 0.920 in pyridine)
4.315 mg. substance: 3.115 mg. H_2O, 10.110 mg. CO_2
3.970 " " : 2.225 " AgI
$C_{24}H_{36}O_8$. Calculated. C 63.68, H 8.02, OCH_3 6.86
Found. " 63.90, " 8.08, " 7.40

Di-p-Bromobenzoate of the Methyl Ester, $C_{24}H_{36}O_8$—The above ester was acylated with *p*-bromobenzoyl chloride in pyridine solution. The di-*p*-bromobenzoate is very sparingly soluble in methyl alcohol from which it crystallizes as long, rectangular plates which melt at 297–298°.

4.350 mg. substance: 2.060 mg. H_2O, 8.920 mg. CO_2
8.690 " " : 4.210 " AgBr
$C_{38}H_{42}O_{10}Br_2$. Calculated. C 55.72, H 5.19, Br 19.53
Found. " 55.92, " 5.30, " 20.62

Diketo Hydroxylactone Dibasic Acid Monomethyl Ester, $C_{24}H_{32}O_9$ *(Formula VIII)*—40 gm. of the above methyl ester, $C_{24}H_{36}O_8$, were dissolved in 300 cc. of 90 per cent acetic acid and, while stirring mechanically, 175 cc. of Kiliani's CrO_3 solution were added slowly. The temperature was kept at 10–15°. After 1 hour, the mixture was diluted and $(NH_4)_2SO_4$ was added. On standing, the diketo acid separated as fine, stout prisms. The collected material was dissolved in dilute Na_2CO_3 solution. The solution was extracted several times with chloroform, which removed a small quantity of neutral impurities. On acidification of the carbonate solution with acetic acid, the acid was slowly reprecipitated. The substance is characterized by its extreme insolubility in the usual solvents. It can be recrystallized from a very large volume of acetone, from which it separates as micro boats which melt at 220–221° with decomposition.

$[\alpha]_D^{21} = +21°$ (c = 0.656 in pyridine)
3.900 mg. substance: 2.460 mg. H_2O, 8.882 mg. CO_2
3.850 " " : 1.950 " AgI
$C_{24}H_{32}O_9$. Calculated. C 62.04, H 6.95, OCH_3 6.68
Found. " 62.11, " 7.06, " 6.69

15.714 mg. of substance were suspended in 1 cc. of alcohol and titrated against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent, 0.339 cc.; found, 0.393 cc. 3 cc. of 0.1 N NaOH were then added and the mixture was refluxed for 4 hours and then titrated back. An additional 0.460 cc. of 0.1 N NaOH were used. During this treatment the resistant methyl ester was only partially saponified.

Dimethyl Ester of the Diketo Acid, $C_{24}H_{32}O_9$—The above acid was esterified in acetone suspension with diazomethane. The ester is easily soluble in the usual solvents. Upon recrystallization by careful dilution of its methyl alcoholic solution it forms flat prisms which soften to a mass of bubbles at 116–118° and then melt at about 180°. For analysis it was dried at 80° and 20 mm.

4.050 mg. substance: 2.660 mg. H_2O, 9.275 mg. CO_2
4.353 " " : 4.275 " AgI
$C_{25}H_{34}O_9$. Calculated. C 62.73, H 7.16, OCH_3 12.97
Found. " 62.46, " 7.35, " 12.98

Dihydroxydilactone Ester, $C_{24}H_{34}O_8$ (Formula XI)—The catalytic reduction of the above diketo acid, $C_{24}H_{32}O_9$, goes on with extreme difficulty. 1 gm. of the acid was finely ground and suspended in a large volume (about 500 cc.) of ethyl acetate. After shaking for several days in an atmosphere of purified hydrogen with 0.5 gm. of Adams and Shriner catalyst, 2 mols of hydrogen were absorbed. During this operation the sparingly soluble starting material gradually dissolved. Complete solution was taken as the sign of completed reaction. The filtrate from the catalyst was washed with Na_2CO_3 solution, which removed the isomer to be described below. The ethyl acetate solution was concentrated to a small volume and chilled. The dilactone separated as fine needles. However, it appeared to be contaminated with a small amount of a persistent impurity which repeated recrystallizations from a variety of solvents did not remove and which caused the carbon figures to come slightly high. This was observed with repeated preparations. In subsequent derivatives, however, this discrepancy was corrected. The substance is sparingly soluble in the alcohols, chloroform, acetone, and ethyl acetate. It melts at 280–281°.

$[\alpha]_D^{27} = +27°$ (c = 0.940 in pyridine)
4.452 mg. substance: 3.090 mg. H_2O, 10.760 mg. CO_2
4.435 " " : 2.968 " " 10.482 " "
$C_{24}H_{34}O_8$. Calculated. C 63.96, H 7.61
Found. " 64.47, " 7.60
" " 64.46, " 7.49

14.592 mg. of substance were suspended in 1 cc. of alcohol and 3 cc. of 0.1 N NaOH and the mixture was refluxed for 2 hours and then titrated back against phenolphthalein. Calculated for 2 equivlents, 0.648 cc.; found, 0.682 cc. The resistant methyl ester was only partly saponified. Upon acidification of the saponification mixture, the original dilactone was recovered.

1.880 mg. substance: 1.285 mg. H_2O, 4.425 mg. CO_2
Found. C 64.19, H 7.65

The Na_2CO_3 extract of the ethyl acetate solution was acidified with acetic acid and concentrated in a vacuum at room temperature to a small volume. Stout rectangular prisms of a neutral substance deposited, which were collected. In contrast to the previous substance it is very soluble in acetone and crystallizes on dilution as fine needles. It decomposes to a solid at 243–244° which melts at 281–282°.

$[\alpha]_D^{22} = +34°$ (c = 0.890 in pyridine)
4.226 mg. substance: 2.830 mg. H_2O, 9.940 mg. CO_2.
Found. C 64.15, H 7.49

Acetate of the Dihydroxydilactone Ester, $C_{24}H_{34}O_8$—0.1 gm. of the isomer melting at 280–281° was refluxed in 5 cc. of acetic anhydride for 30 minutes. The solution was diluted, and, after decomposition of the acetic anhydride, was extracted with chloroform. The chloroform solution was washed free from acid and concentrated to dryness. The residue was recrystallized by careful dilution of its acetone solution. The acetate formed stout prisms and melted at 216–217°.

4.766 mg. substance: 3.145 mg. H_2O, 11.025 mg. CO_2
$C_{26}H_{36}O_9$. Calculated. C 63.38, H 7.37
Found. " 63.09, " 7.38

Sulfite of the Dihydroxydilactone Ester, $C_{24}H_{34}O_8$—0.1 gm. of the isomer melting at 280–281° was simultaneously chilled and covered with 2 cc. of thionyl chloride. Immediate solution occurred with effervescence. After standing 45 minutes, the excess reagent was removed in a vacuum. The residue crystallized as rhombs under methyl alcohol. After recrystallization from methyl alcohol, in which it is sparingly soluble, the substance melts at 229° with decomposition.

4.495 mg. substance: 2.650 mg. H_2O, 9.640 mg. CO_2
10.276 " " : 4.590 " $BaSO_4$
$C_{24}H_{32}O_9S$. Calculated. C 58.02, H 6.51, S 6.47
Found. " 58.49, " 6.58, " 6.28

Hydroxyketodilactone Ester, $C_{24}H_{32}O_8$ *(Formula XIII)*—0.4 gm. of the above dilactone ($[\alpha] = +27°$) was dissolved in 10 cc. of 90 per cent acetic acid and oxidized with 2 cc. of 20 per cent CrO_3 solution. Kiliani's reagent, which contains sulfuric acid, could not be used since it caused dehydration in the process of extracting the product. After 30 minutes the reaction mixture was diluted and after warming the mixture was extracted repeatedly with hot chloroform. The chloroform extract was washed free of acetic acid with hot water and concentrated to dryness. The residue was recrystallized from acetone and formed fine rectangular prisms which melted at 159–160° with decomposition.

$[\alpha]_D^{27} = +21°$ (c = 0.640 in pyridine)
4.382 mg. substance: 2.895 mg. H_2O, 10.290 mg. CO_2
$C_{24}H_{32}O_8$. Calculated. C 64.25, H 7.19
Found. " 64.04, " 7.39

Oxime of the Hydroxyketodilactone Ester, $C_{24}H_{32}O_8$—The oxime was prepared by refluxing the ketone with twice the theoretical quantities of hydroxylamine hydrochloride and sodium acetate in alcohol for 5 hours. It crystallizes from dilute methyl alcohol as irregular platelets which melt at 195–200° after softening at about 175°.

6.175 mg. substance: 0.158 cc. N_2 (27°, 747.3 mm.)
$C_{24}H_{33}O_8N$. Calculated, N 3.02; found, N 2.87

Anhydroketodilactone Ester, $C_{24}H_{30}O_7$ (Formula XIV)—0.15 gm. of the ketodilactone, $C_{24}H_{32}O_8$, was refluxed in 10 cc. of methyl alcohol and 0.5 cc. of HCl (1.19) for 15 minutes. The solution was then boiled down to 2 to 3 cc., and on chilling the anhydro ketone crystallized as hexagonal leaflets. After recrystallization from methyl alcohol it melted at 198°.

$[\alpha]_D^{26} = +68°$ (c = 0.880 in pyridine)
4.427 mg. substance: 2.830 mg. H_2O, 10.805 mg. CO_2
$C_{24}H_{30}O_7$. Calculated. C 66.94, H 7.03
Found. " 66.56, " 7.15

When the dilactone, $C_{24}H_{34}O_8$, was boiled for 30 minutes with methyl alcoholic HCl and concentrated as in the process used above, it was recovered unchanged.

4.428 mg. substance: 3.050 mg. H_2O, 10.440 mg. CO_2
Found. C 64.30, H 7.71

[Reprinted from The Journal of Biological Chemistry, September, 1932, Vol. 97, No. 3, pp. 739–743]

THE ERGOT ALKALOIDS

I. The Oxidation of Ergotinine

By WALTER A. JACOBS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 21, 1932)

Some time ago we became interested in the problem of the structure of the ergot alkaloids. Experiments on the oxidative degradation of ergotinine were already in progress when Barger's monograph[1] came to our attention, in which certain observations of Soltys[2] were discussed. The latter have since been separately published. In this work benzoic acid was obtained on oxidation of each of the alkaloids with permanganate. When nitric acid was used as the oxidizing agent, *p*-nitrobenzoic acid was the only substance recovered.

In our own experiments we have also used nitric acid and regularly obtained *p*-nitrobenzoic acid in addition to small amounts of benzoic acid. Since, however, we have constantly isolated from the reaction mixture a third crystalline substance of more complicated character, we wish to present a preliminary report of these experiments. After oxidation of the alkaloid as later described, benzoic acid, *p*-nitrobenzoic acid, and other substances were removed by ether extraction. The remaining aqueous solution yielded a sparingly soluble, ether-insoluble, substance in a naturally poor yield (6 per cent). The first suspicion that the substance is a nitro derivative was removed by the negative outcome of reduction experiments. It was then found to have retained the methylimino group of the parent ergotinine, the only one of the original 5 nitrogen atoms which remained. The analytical figures indicated a formula $C_{14}H_9O_8N$. Repeated titrations of a number of preparations were in excellent agreement for a substance containing three carboxyl groups. The remaining 2 oxygen atoms have not yet been accounted for. No evidence of the presence of a lactone group could be obtained on boiling with alkali. A methyl indole or perhaps a methyl hydroquinoline nucleus appears to be indicated in this substance. Whether a third, perhaps furane, ring is present cannot be considered until further data are available.

---

[1] Barger, G., Ergot and ergotism, London (1931).

[2] Soltys, A., *Ber. chem. Ges.*, 65, 553 (1932).

Attempts to prepare a methyl ester with diazomethane resulted only in a non-crystalline reaction product. When the tribasic acid was heated with methyl alcoholic hydrochloric acid, only one carboxyl group was esterified with the formation of a *mono-methyl ester*. The acid is readily oxidized by permanganate. Owing to the difficulty of procuring sufficient material, a report of this reaction will be left to a later communication.

Further work on the oxidative degradation of the ergot alkaloids is in progress.

EXPERIMENTAL

In the oxidation of ergotinine with nitric acid a number of experiments was made in which the procedure was modified somewhat in each case according to the objective. The following experiments are reported as they were actually performed.

2 gm. of ergotinine were treated with 50 cc. of HNO_3 (1.4 sp. gr.). A vigorous reaction occurred. The mixture was then heated on the water bath for 18 hours. On dilution a definite odor of isobutyric acid was in evidence. The mixture was distilled over into dilute alkali but the amount of volatile material which was collected was too small in amount for study.

The orange-red acid solution which remained was evaporated on the bath to about 50 cc. On standing, clusters of pale yellow platelets separated. These were collected on a Jena funnel with nitric acid and then washed with water. The yield was 70 mg. After recrystallization from dilute acetone lustrous leaflets of *p*-nitrobenzoic acid separated, which melted at 235–236°, and showed no depression when mixed with *p*-nitrobenzoic acid.

3.815 mg. substance: 1.030 mg. H_2O, 7.052 mg. CO_2
3.870 " " : 0.292 cc. N (758.7 mm., 24°)
$C_7H_5O_4N$. Calculated. C 50.28, H 3.02, N 8.39
Found. " 50.41, " 3.02, " 8.66

The mother liquor of the above crude *p*-nitrobenzoic acid was concentrated practically to dryness and left a considerable resinous residue. This was treated with water and after standing additional crystalline material mixed with a brown-red resin deposited. After collection with water all that could be recovered from this material was an additional small quantity of impure *p*-nitrobenzoic acid.

The mother liquor from this second crude fraction was diluted somewhat and then extracted in a continuous extractor for 24 hours with ether. The aqueous layer which remained on concentration gave a small amount of the crystalline acid which softened above 280° after preliminary darkening. This substance was obtained in larger yield in later experiments as follows:

5 gm. of ergotinine were treated with a mixture of 120 cc. of HNO_3 (1.4) and 675 cc. of water and slowly distilled in an all-glass apparatus. The alkaloid rapidly became superficially colored a dark greenish brown and remained at first mostly as an undissolved resin. Shortly, a copious CO_2 formation could be readily detected with barium hydroxide. As the concentration of acid increased, the mixture became gradually a deep brown-red and small amounts of volatile material which crystallized in the receiver were carried over. The odor of this suggested somewhat *p*-nitrotoluene but the amount which could be collected was too small for identification. On reaching a stronger concentration of acid, the alkaloid had mostly dissolved and red fumes began to appear. At this point 400 cc. of water were again added and the distillation was resumed. On reaching the point of strong acid the dilution and concentration were repeated. These operations were repeated several times more. By this time the appearance of volatile solid material had practically ceased. Finally, the mixture was boiled down to the point of strong nitric acid when the odor of benzoic acid became evident in the distillate. At this point the operation was interrupted. The combined distillates were treated as given further on.

The deep brown-red acid solution was then concentrated *in vacuo* in an all-glass apparatus to remove the excess of nitric acid. The residue was dissolved in about 75 cc. of hot water. On standing, a small amount of red tar deposited. The supernatant liquor was decanted and concentrated to smaller volume. A crop of somewhat sulfur-yellow crystalline aggregates slowly deposited. After collection, this was recrystallized by solution in 100 cc. of boiling water. On standing, faintly yellow aggregates of minute prisms slowly separated. The yield was 0.14 gm. The substance sintered above 280° after preliminary darkening. The mother liquor of this last recrystallization gave on concentration a second crop which was collected with water. It was found to be contaminated with considerable ether-soluble material and was therefore digested with ether and collected with this solvent. The yield was 50 mg. of this second crop which also softened above 280°. The ether washings on concentration gave 40 mg. of crystalline material which melted at 234° and proved to be *p*-nitrobenzoic acid.

The fact that the above high melting substance is practically insoluble in ether was then made use of in later experiments. 5 gm. of ergotinine were oxidized as previously. After final removal of the excess of nitric acid by concentration under diminished pressure the residue was dissolved in about 250 cc. of water. The brown-red mixture which contained suspended resin was placed in an extractor and continuously extracted with ether for 2 days, by which time no more color passed into the ether layer. The aqueous layer which was still brown-red and contained some suspended resin, was filtered and concentrated to small volume. Successive crops of crude crystalline acid were obtained, all of which softened at about 280–285°. The yield was 0.3 gm.

This was dissolved in 300 cc. of boiling water and cleared with norit. The filtrate was a faint straw-yellow. On concentration to about half and addition of dilute HCl the acid separated as almost colorless needles. It does not possess a characteristic melting point. On rapid heating it darkened above 265° and sintered together at about 290°, but did not melt even up to 350°. The substance is less soluble in dilute acid than in water. It is very sparingly soluble in the usual organic solvents. Its solution in dilute alkali, which is a pale yellow, exhibits a slight fluorescence. The substance gives only a faint pine splinter test.

14.322 mg. of substance were suspended in a few cc. of water and titrated against phenolphthalein with 0.1 N NaOH. Calculated for 3 equivalents, 1.346 cc.; found, 1.345 cc.

4.458 mg. substance: 1.085 mg. H_2O, 8.626 mg. CO_2
5.287 " " : 0.201 cc. N (759.2 mm., 25°)
4.760 " " : 3.275 mg. AgI

$C_{14}H_9O_8N$. Calculated.	C 52.65,	H 2.84,	N 4.39,	NCH_3	9.10
Found.	" 52.77,	" 2.72,	" 4.36,	"	8.51

The attempt to prepare a crystalline neutral methyl ester from the acid with diazomethane was unsuccessful. Only resinous material resulted. A monomethyl ester was prepared as follows: The acid was heated in a sealed tube for 18 hours with 50 parts of absolute methyl alcohol which contained 4 per cent of HCl. The crystalline powder was collected with methyl alcohol in which it is sparingly soluble. On heating, it behaves like the parent acid with no distinct melting point. It darkened above 260° and sintered at about 285° but did not melt below 350°.

4.050 mg. substance: 1.150 mg. H_2O, 7.970 mg. CO_2
3.700 " " : 2.555 " AgI
$C_{15}H_{11}O_8N$. Calculated. C 54.04, H 3.33, OCH_3 9.31
Found. " 53.67, " 3.18, " 9.13

The combined distillates obtained during the nitric acid oxidations were repeatedly extracted with ether. The latter was washed once with a little water and then extracted with dilute sodium hydroxide solution. The alkaline solution was acidified with sulfuric acid and as there was a pronounced odor of isobutyric acid the attempt was made to distil this into dilute alkali. The amount which distilled over, however, proved to be inadequate for definite identification. During this distillation a small amount of crystalline material was also carried over, which was obviously benzoic acid. This substance was also recovered from the acid solution which remained after the distillation of the volatile material. On cooling, a relatively very small amount crystallized. After recrystallization from ligroin it melted at 120–121°.

4.493 mg. substance: 1.998 mg. H_2O, 11.340 mg. CO_2
$C_7H_6O_2$. Calculated. C 68.82, H 4.96
Found. " 68.83, " 4.98

The ether solution which retained neutral material after the above extraction with alkali gave on concentration a small partly crystalline residue. The amount was too small for identification.

[Reprinted from SCIENCE, September 2, 1932, Vol. 76, No. 1966, pp. 219–220]

THE FUNCTIONAL CHARACTERISTICS OF NINE RACES OF FIBROBLASTS

BY RAYMOND C. PARKER

(From the Laboratories of The Rockefeller Institute for Medical Research)

Common connective tissue cells, or fibroblasts, being the first to be isolated in pure cultures, have served as material for a vast array of studies from which much valuable information has been gained concerning the structural and functional properties of cells in general and of these cells in particular. For convenience, and in order that comparable results might be obtained by the various investigators, the material most generally used has been the original strain of fibroblasts isolated over 20 years ago, by Carrel, from the embryonic chick heart. Hence, the properties of these cells have become very well known. Some years ago, it was demonstrated, however, that functionally different cell strains, each of them possessing all the structural features commonly attributed to fibroblasts, could be isolated from various tissues of the same organism.[1] This disclosure, which resulted from a study of the diverse manner in which four different strains of fibroblasts reacted to a given nutritional régime, clearly indicated the error involved in confining classifications and definitions of cells types to purely morphological characters, without at the same time taking into account their physiological properties. It is believed that the additional information to be reported here will not only strengthen this view-point, but will also show the importance of enlisting as many of the characteristics of these cells as may be revealed, and of subjecting each to careful and systematic analysis, before attempting to explain the biological significance of any one of them.

Several series of experiments have recently been made in which a varying number of cell strains were isolated simultaneously from different tissues and organs of the same chick embryo and, from the very beginning, subjected to conditions which were as identical as it was possible to make them. The procedures employed were the usual ones involving the flask techniques. Very soon after the tissues were removed from the organism, the cell population was rendered uniform by continued selection of only the marginal areas of outgrowth at the time of transfer. Then, by comparing these strains with one another, and with strains from similar series derived from embryos of the same age, it was possible to detect any outstanding properties manifested under the conditions of the experiment. The particular series that has been selected for the purpose of the present communication was composed of nine strains of fibroblasts isolated from a 17-day-old chick embryo and cultivated for ten passages (56 days) on a medium consisting of chick plasma, chick embryonic tissue juice and Tyrode solution. These strains were derived from the following tissues and organs: the wall of the dorsal aorta, the periosteum of bone, the perichondrium of cartilage, the wall of the ventricle, the wall of the proventriculus, the muscles of the lower limb, the kidney, the thyroid and the testis.

---

[1] R. C. Parker, *Arch. f. exper. Zellforschung*, 8: 340, 1929.

Aside from making a comparative study of the rate of growth of the various tissues over the entire period of cultivation, tests were carried out from time to time to determine the relative amount of free acid that accumulated in the medium. For this purpose, a dilute solution of phenol red was added to the medium of each flask, after which the hydrogen-ion concentration was adjusted by introducing into the flask a gas mixture of O_2, CO_2 and N, these being combined in such proportions as to produce a temporary acidity of pH 7.8. The changes produced in the various cultures could then be read at 24 or 48 hour intervals by comparing them with a standard series of flasks of known pH values. Other experiments were designed to test the ability of the various races to survive and grow in an abnormally acid medium. This condition was also brought about by subjecting the cultures to an atmosphere composed of O_2, CO_2 and N, but this time the mixture contained a greater concentration of Co^2 than that which had been used to establish a pH of 7.8. Thus it was found that the various tissues differed not only in their rate of growth and in the amount of acid that accumulated in the medium in which they were cultivated, but also in their ability to survive and grow in a medium in which the hydrogen-ion concentration was artificially increased. In addition, they showed marked variations in the extent to which they were able to digest fibrin. The accompanying diagram (Fig. 1) shows the relative growth rates displayed by these strains in their eighth passage. It will be seen that the fibroblasts derived from the heart muscle possessed the lowest growth energy of any of the series, whereas the fibroblasts from skeletal muscle showed the highest. In this respect, these two strains represented the extremes. Furthermore, these rapidly growing fibroblasts from skeletal muscle liberated a large amount of acid into the medium and were able to withstand a medium of high acidity. There was, however, very little digestion of fibrin. Fibroblasts from the proventriculus and the kidney, on the other hand, produced little acid, but rapidly digested the fibrin of the clot. The same was essentially true of

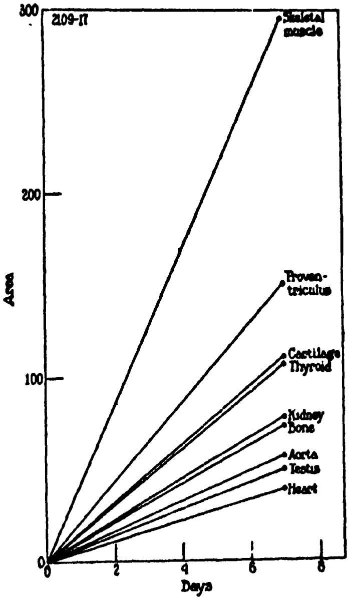


FIG. 1. Diagrammatic representation of the rate of growth of nine strains of fibroblasts isolated from a 17-year-old chick embryo and subjected to the same treatment from the beginning; 8th passage.

fibroblasts derived from the thyroid, although in this case the colonies grew extremely thin, much more so than has ever been observed for other races of fibroblasts. The fibroblasts from the aorta were characterized by a large production of acid. Although their rate of multiplication was much slower than that of fibroblasts from cartilage, thyroid, kidney or the proventriculus, they produced much more acid than any of these. In spite of this, however, they grew poorly in an acid medium.

These results indicate that the common connective tissue cell, or fibroblast, does not occur throughout the organism as a separate and distinct type. Fibroblasts as a group include many different cell types. Just, for example, as the *milieu intérieur* of the thyroid is different from that of the kidney, so also are the connective tissue cells which they harbor and nourish. Hence, it seems reasonable to assume that there are as many types of fibroblasts in the body as there are tissues and organs. These various cell types were originally endowed with identical properties and potencies by virtue of a common ancestry. But as they became integral parts of developing tissues and organs, they became more and more divergent, with the final result that, when separated from the organism, they retained those qualities that they had progressively acquired as an expression of the special localized conditions under which they had lived prior to their isolation.

[Reprinted from SCIENCE, November 11, 1932, Vol. 76, No. 1976, pp. 446–447]

THE STABILITY OF FUNCTIONALLY DISTINCT RACES OF FIBROBLASTS

BY RAYMOND C. PARKER

(From the Laboratories of The Rockefeller Institute for Medical Research)

It has already been shown[1] that common connective tissue cells, or fibroblasts, do not occur throughout the organism as a separate and distinct type. Fibroblasts, as a group, comprise many different cell types which, according to their origin, can be distinguished by the physiological properties manifested under conditions of cultivation *in vitro*. They differ with respect to the growth energy that they exhibit in a given medium, the amount of acid liberated into that medium, their ability to digest fibrin, etc. Thus, although morphologically similar, a fibroblast from skeletal muscle is as different from a heart muscle fibroblast as a pigment cell of iris epithelium is different from a colloid-producing cell from thyroid epithelium. Furthermore, it has been found[2] that fibroblasts originating from embryos of different ages show different nutritional properties, even when the cells are removed from the same part of the embryo. For example, cell strains derived from a 20-day-old embryo may show, under the same experimental conditions, a rate of cell multiplication that is either higher or lower than that exhibited by similar strains isolated from a 10-day-old embryo, depending upon the particular organ or tissue from which the strains are obtained. In the present communication, it will be shown that, after separation from the body, each cell type retains indefinitely the properties possessed at the moment of isolation.

Two groups of experiments have been made. The first group was designed to test the permanence of differences due to spatial relationships, that is, those determined by the particular organ or tissue supplying the strains; the second, to test the permanence of differences of a temporal nature, or those conditioned by the age of the embryo from which the tissues are taken. The material used in connection with the first set of experiments consisted of two strains of fibroblasts derived from a 13-day-old chick embryo, one being obtained from the ventricle of the heart, the other from the periosteum of bone. Both strains were subjected to the same treatment for 18 passages (97 days), during which time they were cultivated in an appropriate mixture of chick plasma, chick embryonic tissue juice and Tyrode solution. At the 19th passage, the two strains were placed in media of different composition, the heart fibroblasts being cultivated in a medium containing much more than the usual amount of embryonic tissue juice, while the fibroblasts from the periosteum were subjected to a medium in which the percentage of tissue juice was greatly diminished. This treatment, which was continued for 4 passages (26 days), resulted in an increased rate of cell multiplication on the part of the heart fibroblasts with a corresponding retardation in the case of the bone fibroblasts. At the end of four passages, when the heart fibroblasts were dividing at a much higher rate than the starved bone fibroblast, the two strains were again placed under the same nutritional régime. Almost immediately, they reverted to their original rate of division, and after three passages on a common medium they both displayed the same characteristics that had distinguished them before it was attempted to alter their properties by subjecting them to diverse media.

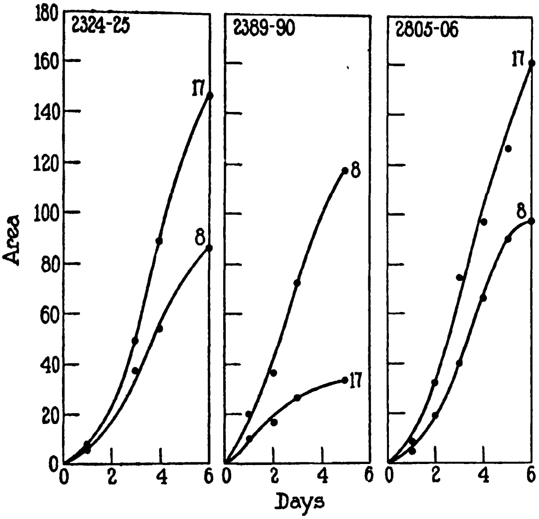
---

[1] R. C. Parker, SCIENCE, 76, 219, 1932.

[2] Unpublished experiments.

The second group of experiments, being designed to test the permanent nature of nutritional properties determined by the age of the embryo from which the fibroblasts are derived, were carried out in much the same manner as those described above. Two cell strains were isolated simultaneously from skeletal muscle removed from the lower limbs of 8- and 17-day-old chick embryos. Both strains were cultivated under the same environmental conditions. The cell strain derived from the 17-day-old embryo showed a rate of cell multiplication that was consistently higher than that manifested by the strain derived from the younger embryo. The degree of difference was as great as that found to exist between the heart and bone fibroblasts referred to above. The accompanying diagram shows the division rate of the two strains in their fourth passage (Fig. 1). At the previous transfer, one half of each culture comprising the two strains was placed in an experiment in which the 8-day strain was given access to a larger quantity of embryonic tissue juice than it had previously received, whereas the 17-day strain was placed in a medium containing much less than the usual amount. The result was similar to that obtained with the heart and bone fibroblasts. The rate of cell multiplication of the 8-day strain, that had originally been much lower than that of the 17-day strain, now became the greater of the two (Fig. 2). After this treatment had been continued for two successive periods of cultivation, they were replaced in media of the same composition. The original characteristics reappeared immediately (Fig. 3), and continued to persist until the termination of the experiment, at which time the strains had been cultivated *in vitro* for a total period of 75 days.

FIG. 1 FIG. 2 FIG. 3



FIGS. 1–3. Curves showing the rate of growth of two strains of fibroblasts isolated simultaneously from the musculature of the lower limb of an 8- and a 17-day-old chick embryo, respectively, and subjected to identical treatment from the beginning until the 26th day. Fig. 1 shows the last 6 days of this treatment. Fig. 2 shows the rate of growth of the same strains for the second of two 5-day periods, during which time the 8-day strain was subjected to more than the usual amount of food substances, and the 17-day strain to less than the usual amount. Fig. 3 shows the rate of growth of the same strains after the previous treatment had been discontinued and both strains had again been cultivated on the same medium for 29 days.

It is clear that the properties characterizing the different races of fibroblasts are real and persistent. They are retained by the cells in an unaltered state in spite of profound alterations in their environment. It is therefore erroneous to suppose that cells removed from the organism revert to an embryonic or indifferent type. While the properties that the cells manifest under various conditions of cultivation outside of the organism are, in part, an expression of the nutritional conditions under which they are placed, they likewise depend upon their origin and upon the age of the animal from which they are removed. The organism is able to effect progressive and irreversible changes in the constituent cells. But when the cells are released from the influence of the organisms, they remain permanently stamped with the characters imposed upon them by the peculiar conditions that had existed in the tissues and organs of which they formed a part.

[Reprinted from ERGEBNISSE DER ENZYMFORSCHUNG, 1932, 1, pp. 302–324]

CRYSTALLINE PEPSIN[1])

BY JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

CONTENTS

1. Introduction 197
2. Preliminary Experiments 198
3. Isolation of the Crystalline Enzyme 200
4. General Properties and Analysis of the Crystals 203
5. Evidence Concerning the Purity of the Crystalline Material 206
6. Conclusions as to the Purity of the Preparation 222
7. Proteolysis Is a Homogeneous Reaction 224
8. Summary 224

1. INTRODUCTION

Enzymes, in many respects, are connecting links between living and inanimate matter since their action is analogous to inorganic catalysts, although the enzymes themselves are found only in living organisms. As catalysts they increase the rate of one or more specific reactions and so act as directive agents for the reactions occurring in the organism. This directive property is undoubtedly essential for the existence of living cells. As a consequence of these properties the study of enzymes has been of interest to both chemists and biologists and has resulted in a great increase in the knowledge of their mode of action. The results of attempts to isolate the enzymes in pure form, however, have been singularly unsuccessful. There seems to be no convincing evidence that any enzyme has been obtained in the pure state; and only one, the urease described by Sumner[2]), has been previously obtained in crystalline form.

In the case of pepsin, however, preparations have been obtained by several workers, which had fairly constant activity, so that this enzyme seemed a favorable one for isolation experiments. Pepsin has the further great advantage over most other enzymes in that it is comparatively stable, and that active preparations may be purchased in quantity thus avoiding the troublesome and laborious first steps in purification. In this connection it may be noted that crude material equivalent to more than 20,000 pig stomachs was used in the work reported in this paper. This would obviously have been impossible had it been necessary to carry out the entire process in the laboratory. The crystallization of the material was possible only because of the fact that large quantities of material were used. Crystallization occurs only from concentrated solutions and concentrated solutions cannot be prepared and handled, with small quantities of material.

---

[1]) J. H. Northrop, Science **69**, 580. 1929; J. gen. Physiol. **13**, 739, 767. 1930 (This paper contains detailed description of the experimental procedure).

[2]) J. B. Sumner, J. biol. Chem. **69**, 435. 1926.

2. *Preliminary Experiments*

A number of methods have been proposed for the purification of pepsin such as precipitation with safranin¹), etc., fractionation by various adsorbents and precipitation by dialysis from acid solution (Pekelharing²). These and several other methods were tried and more or less active preparations obtained. The results with Pekelharing's method seemed the most encouraging, however, since the loss of activity was less and there was some indication that a constant activity was reached. This result had been reported by Pekelharing and also by Fenger, Andrew and Ralston³) using a similar method. It was found, however, that the dialysis could be dispensed with and the process made more rapid and efficient by solution with alkali and subsequent precipitation with acid, after a preliminary precipitation with half saturated $MgSO_4$ or $(NH_4)_2SO_4$. The amorphous material so obtained contains about half the activity present in the original material and is 3 to 6 times as active as measured by the liquefaction of gelatin and about 5 times as active as measured by the digestion of casein or by the rennet action on milk.

---

¹) H. R. Marston, Biochem. J. **17**, 851. 1923.

²) C. A. Pekelharing, Z. physiol. Ch. **22**, 233. 1896.

³) F. Fenger, R. H. Andrew and A. W. Ralston, J. biol. Chem. **80**, 187. 1928.

*Determination of Activity*1).—The rate of digestion of proteins by pepsin may be followed by determining the increase in carboxyl groups or amino nitrogen, or by the decrease in protein nitrogen or by the changes in viscosity. The increase in carboxyl groups probably represents the best measure of the progress of the reaction and has been used as the basis of the activity units used in this paper.

The most widely used unit of enzyme activity in general is that suggested by Euler2) and is equal to the velocity constant of the reaction divided by the grams of enzyme. Theoretically this is undoubtedly correct since the activity of a catalyst can be expressed only as a velocity constant. Many enzymes however do not give a velocity constant independent of the substrate concentration but instead the velocity constant decreases as the substrate concentration increases. This is especially true of pepsin and in addition the reaction does not follow any simple reaction rate. The end point of the reaction, which must be known in order to calculate the velocity constant, is also very difficult to determine. It is not practical therefore to use the velocity constant as a measure of activity of pepsin.

The activity of pepsin may conveniently be defined as the milliequivalents of carboxyl groups liberated per mole or gram of enzyme per minute at 35,5°C., optimum p_H, and 5 per cent substrate concentration. Theoretically it would be better to use that substrate concentration at which the rate of reaction is a maximum, but experimentally this is difficult, owing to the high viscosity of such concentrated solutions. The enzyme concentration used should be in the range in which the activity is proportional to the enzyme concentration. In order to determine the activity in this way the rate of hydrolysis of casein, gelatin, edestin, and denatured egg albumin at p_H 2,0 to 2,5, was determined by means of the increase in formol titration3).

The following abbreviations are used in this paper:

PU = proteolytic units = milliequivalents carboxyl groups per minute.
$[PU]_{\text{gm.}}$ = proteolytic units per gram.
$[PU]_{\text{gm.}}^{\text{cas. F.}}$ = proteolytic units per gram as determined by cas. F. method, etc.

Repetition of this procedure gave products of increasing activity as measured by the liquefaction of gelatin and apparently this activity could be increased indefinitely. Several samples were obtained which were 100 times as active as the original preparation. They were also more unstable so that each succeeding precipitation was accompanied by a larger and larger percentage loss until finally no more active

---

1) J. H. Northrop, Science **69**, 580. 1929; J. gen. Physiol. **13**, 739, 767. 1930 (This paper contains detailed description of the experimental procedure).

2) H. Euler, Chemie der Enzyme, 3rd edition, Part 1, p. 390, Bergmann, München 1925.

3) J. H. Northrop, J. gen. Physiol. **9**, 767. 1926.

material remained. When the activity of the various fractions was determined by the rate of hydrolysis of casein or by the rennet action on milk, however, it was found that the activity increased until it reached about 5 times that of the crude preparation and then remained constant instead of increasing as did the gelatin liquefying power.

This difference in the activity of amorphous preparations as determined by gelatin and by casein, edestin or egg albumin was very puzzling and it was thought for a time that it might be due to the physical state of the material, as had been suggested by Fodor and others. This idea received confirmation from the fact that these abnormally active amorphous preparations, on crystallization yielded crystals having normal activity. However, the reverse experiment could not be performed. The activity of material which had been crystallized several times, could not be changed, although a large number of attempts were made to do so. It was eventually found[1]) that the crude material contains an enzyme which is extremely active in the liquefaction of gelatin but has little or no effect on casein, edestin, egg albumin, nor on coagulation of milk. The crystalline pepsin may be freed from this enzyme by two or three crystallizations. The "gelatinase" has been further purified until it is nearly one thousand times as active as the crude preparation. It is apparently also a protein although there is as yet no evidence that it has been obtained in pure form.

This was the result reported by Pekelharing and also by Fenger, Andrew and Ralston. This material appeared to be protein, as previous workers had found, and was reasonably stable. Efforts were therefore made to isolate this protein in crystalline form.

3. *Isolation of the Crystalline Enzyme*

It was noticed that the precipitate which formed in the dialyzing sac when the procedure of Pekelharing was followed appeared in more or less granular form and filtered rather easily as though it were on the verge of crystallization. This precipitate dissolved on warming the suspension and it was eventually found that it could be induced to crystallize by warming to 45°C., filtering, and allowing the filtrate to cool slowly. The crystals so obtained were regular hexahedra and showed a tendency to grow in clusters especially when appearing from more acid solutions. They are remarkably similar to the urease crystals pictured by Sumner and differ only in that they have a hexagonal base while the urease has an octagonal base. On one occasion a few crystals with truncated pyramids were obtained. They had the same activity and optical activity as the usual form. The crystals showed positive double refraction and were optically active in solution. They possessed proteolytic activity, when dissolved, equivalent to 5 times that of the U. S. P. 1 to 10000 pepsin as measured by hydrolysis of casein, and 2,5 times as measured by the liquefaction of gelatin.

---

[1]) J. H. Northrop, J. gen. Physiol. 1931.

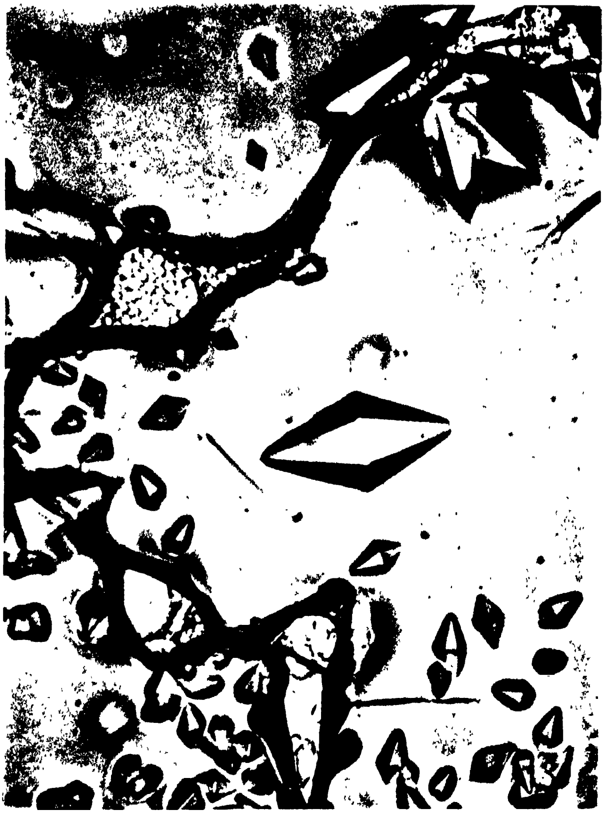


FIG. 1. Crystalline pepsin

a) *Improved Method for the Preparation of the Crystals.*— The isolation of the crystals in bulk by the above method was difficult owing

TABLE I

Preparation of Crystalline Pepsin

Procedure	Activity per gm. dry weight-method			
	Gel. V.	Cas. S.	Rennet	E. A. F.
500 gms. Parke, Davis pepsin U. S. P. 1:10000 dissolved in 500 ml. H_2O and 500 ml. 1 Normal H_2SO_4 added. 1000 ml. saturated $MgSO_4$ added with stirring. Solution filtered through fluted paper (S. and S. No. 1450½) and then with suction. This precipitate must not be allowed to stand at room temperature more than about 24 hrs. Filtrate discarded.	0,075	2,5	2,5	4,5
Precipitate 1. Wash twice with equal volume ⅔ saturated $MgSO_4$, filter with suction. Filtrate discarded.				
Precipitate 2. Stir with water to thick paste and M/2 NaOH run in until complete solution. (Great care must be taken to avoid local excess of NaOH. pH never more than 5,0.)	,15	7,5	7,5	7,0
M/2 H_2SO_4 added with stirring until heavy precipitate forms, (pH about 3,0), 3 to 6 hrs. at 8°C., filter with suction.				
Filtrate discarded.	,15	10,0	10,0	8,0
Precipitate 3. Stir with H_2O to thick paste at 45°C., M/2 NaOH added carefully until precipitate dissolves, (filter if cloudy and discard precipitate). Beaker containing filtrate placed in a vessel containing about 4 liters of H_2O at 45°C. inoculated and allowed to cool slowly, cooling should require 3 to 4 hrs. and heavy crystalline precipitate should form at about 30 to 35°. Solution kept at 20°C. for 24 hrs. Thick crystalline paste, filter with suction.				
Precipitate 4. Wash with small amount of cold H_2O and then with ½ saturated $MgSO_4$ and store under saturated $MgSO_4$ at 5°C.	,17	14,0	[14,0]	9,0
Filtrate. M/2H_2SO_4 added to pH 3,0, amorphous precipitate filtered off and treat as Precipitate 3.				

TABLE I—*Concluded*

Recrystallization

Method 1.	Crystalline paste filtered with suction on large funnel so as to form a thin layer of crystals and washed 3 times with cold M/500 HCl. Filter cake stirred to a paste with ½ its weight of water, the suspension warmed to 45° and M/2 NaOH run in slowly with constant stirring until the precipitate dissolves ($p_H < 5{,}0$). M/2 H_2SO_4 is then run in until the solution is faintly turbid, a few crystals added and the solution allowed to cool slowly as before. A heavy crop of crystals should separate in about 24 hrs. The suspension is then warmed to 45° again and more H_2SO_4 added until the p_H of the suspension is about 3,0. It is then allowed to cool slowly again, and filtered after 24 hrs. The crystals may be washed with M/500 HCl until free of SO_4. Ammonium sulphate may be used in place of $MgSO_4$. Sodium acetate may be used in place of sodium hydroxide.
Method 2.	Crystals dissolved with NaOH and treated as described for Precipitate 2.

to the dialysis. It was found that this could be avoided and the purification carried out as outlined above for the amorphous preparations except that the acid precipitate was dissolved at 45°C. in concentrated solution. On inoculation, this solution set to a solid paste of crystals. Table I is an outline of the method as finally developed.

4. *General Properties and Analysis of the Crystals*

The material prepared in this way has the general properties of a protein. It is coagulated by heat, precipitated by saturation of the solution with $MgSO_4$ or $(NH_4)_2SO_4$ and gives a strongly positive xanthoproteic test. The Molisch test is negative. The crude material contains a large amount of yellowish pigment which is removed with difficulty. It may be largely removed by reprecipitation with $MgSO_4$ and becomes less as the material is recrystallized. All the preparations, however, give a slightly yellowish solution when dissolved although the dry crystals are pure white after several crystallizations. There is no relation between the activity and the color. Analysis of the material gave the results shown in Table II.

The crystals are difficult to dissolve after drying and are best kept under saturated $MgSO_4$ at 5°C. They are instantly inactivated by alkali in solution and lose activity slowly in acid solutions. The inactivated material is digested by the remaining active material and a large amount of tyrosin crystallizes out. This process also occurs slowly in the icebox so that the crystals on standing become mixed with non-protein material that is not precipitated by salt nor by heat. The crystals can be freed from this soluble material by thorough washing with H_2O or by recrystallization. When freshly prepared in this way 98 to 99 per cent of the nitrogen is precipitated from solution by heating rapidly to boiling at p_H 3 with sulphuric acid and Na_2SO_4, by saturation with $MgSO_4$ or $(NH_4)_2SO_4$, by the addition of alkali and subsequent neutralization, or by heating with 10 per cent trichloroacetic acid.

TABLE II

Analysis. Dried at 60° in Vacuo for 24 Hrs.

Method..........	Dumas	Kjeldahl	Van Slyke							Precipitation with $BaCl_2$
	Total N	Total N	Amino N	C	H	Cl	S	P	Ash	$SO_4^{=}$
Per cent dry weight	15,5 15,3	15,15	0,80	52,3 52,6	6,66 6,64 6,70	0,23 ,20	0,88 ,82	0,078	0,40 ,55	0

TABLE III

Activity and Composition of Various Preparations of Pepsin, Crystallized Once

Preparation	1	2	3-5	5-10	10-12	12-20	22
$[PU]^{gel. V.}_{gm.}$	0,17	0,18	0,16	0,154	0,16	0,14	0,17
Per cent N	15,2	15,1	15,1	15,3	15,2	15,0	15,1
$[\alpha]^{22°}_{D}$ p_H 4,5	−70	−72	−74	−73	−74	−71	−70

a) *Constant Activity of Various Preparations.*—About 2 kg. of the crystals were prepared from six different lots of the commercial preparation during the course of this work and have all had the same percentage of nitrogen and the same activity within the experimental error. A summary of these properties of the various preparations is given in Table III. The preparations in some cases were combined at the final crystallization.

The relative activity with various substrates and various methods is shown in Table IV. The figures are the average of 6 to 10 determinations with different enzyme preparations. All crystalline preparations tested yielded the same result with these methods but amorphous preparations frequently gave much higher activity as measured by gelatin hydrolysis and lower activity as measured by egg albumin, while the activity as measured by casein hydrolysis remained constant. On recrystallization of these abnormal amorphous precipitates the activity always returned to the characteristic value for the crystalline material, shown in the table.

TABLE IV

Activity of Various Enzyme Preparations

Enzyme	Substrate	Method	Activity milliequivalents per gm. per min. = $[PU]_{gm.}$	Milliequivalents per millimole or equivalents per mole per min. = $[PU]_{mole}$
Crystalline pepsin	Casein	Formol	14 ± 0,5	500
		Solution	[14]	
		Rennet action	[14]	
	Gelatin	Formol	,17 ± 1	6
		Viscosity	[,17]	
	Edestin[1])	Formol	28 ± ,8	1000
	Egg albumin	Formol	9 ± ,5	320
U. S. P. pepsin 1:10,000	Casein	Formol	2.5	
		Solution	[2,5]	
		Rennet action	[2,5]	
	Gelatin	Formol	,075	
		Viscosity	[,075]	
	Egg albumin	Formol	4,5	

[1]) The activity as determined by edestin was found to vary somewhat with different edestin preparations. The other values were reproducible with various preparations of the proteins.

The activity compared to other enzymes is less than that reported by Sumner for urease, on a weight basis and much less than for several other enzymes but comparison should be made on the basis of activity per mole rather than per gram of enzyme.

5. *Evidence Concerning the Purity of the Crystalline Material*

a) *Constant Activity and Composition on Repeated Crystallization.*—The work reported above shows that a crystalline protein having proteolytic activity may be obtained in quantity from commercial preparations of pepsin. The question of interest is whether this proteolytic activity is a property of the protein molecule or of another molecular species associated with the protein. The usual criterion for the purity of a substance is constant composition and properties after repeated recrystallization. Unfortunately in the case of protein the melting point, which is the most sensitive test of purity cannot be used since proteins decompose before melting. Of the other properties, the proteolytic activity, optical rotation and percentage of nitrogen and phosphorus were considered the most significant and these properties were determined on preparations obtained from a series of seven successive crystallizations. A summary of the results of this experiment is shown in Table V. There is no perceptible drift in any of the properties determined and therefore no indication that the material can ever be separated into fractions by crystallization under these conditions. This shows that the composition of the crystals is independent of the concentration and quantity of the solution from which they are formed.

TABLE V

Summary of Recrystallized Pepsin

Crystallization No. and color	Quantity of Crystals	$[PU]^{\text{gel. V.}}_{\text{gm.}}$	$[PU]^{\text{cas. S.}}_{\text{gm.}}$	N	P	$[\alpha]$ $P_H5{,}0$	Formol per gm.
	gm.			*per cent*	*per cent*		*ml.* 0,1 NaOH
1. Dark brown...........	50	0,18	14,0	14,85	0,078	−70	14,0
2. Brown.................	22	,17	14,5	15,0	,080	−70	12,5
3. Yellowish.............	15	,15	15,0	15,14	,075	−68	12,5
4. Slightly yellow fine powder................	9	,16	14,5	15,16	,076	−67,6	11,0
5. Nearly white..........	5	,16	14,8	15,13	,077	−68	11,0
6. " "	3	,14	14,5		,073	−72	11,0
7. " "	,5	,17	15,0	15,15	,080	−72	12,0
Original preparation.......		,075	2,5				

Several systems could give this result[1]).

1. A mixture of two or more substances present in amounts which are proportional to their solubilities.
2. A solid solution of such composition as to have a minimum solubility. This would correspond to a constant boiling point mixture of miscible liquids.
3. A solid solution of two or more substances having nearly the same solubility.
4. A pure substance.

The various possibilities noted above all depend on some definite relation between the composition of the mixture and the solubilities of the hypothetical components. If this relation were changed, the composition would be changed (except in the case of allotropic modifications). The possibility of such a relation would be greatly reduced if the recrystallization were carried out in a number of different solvents in which the solubility was different. It is quite possible that a mixture of constant composition might be obtained from one solvent just as constant boiling mixtures are frequently found, but it would be expected that the composition of the mixture would vary with different solvents just as the composition of constant boiling mixtures varies with the pressure. If it could be shown therefore that the material retained its constant proteolytic power and composition after recrystallization from a number of different solvents, it would be excellent proof that it was a pure substance. Unfortunately, however, it is not possible to crystallize the material from a series of solvents, but the same result may be obtained by studying the solubility in several solvents.

b) *Results of the Solubility Determinations.*—The values for the solubility obtained with different amounts of precipitate in various solvents are shown graphically in Figs. 2 to 6.

In order to save space the scale representing the total amount of nitrogen per milliliter of suspension has been changed to a logarithmic one in some of the curves after the amount has become large as compared to the amount in solution. Since the solubility is found to be independent of the amount of precipitate this procedure does not affect the shape of the curve.

---

[1]) J. H. Northrop and M. Kunitz, Science, **73**, 262. 1931.

The soluble nitrogen per milliliter of solution, the proteolytic activity per milliliter of solution as determined by the digestion of casein or gelatin $[(\mathrm{PU})_{\mathrm{ml}}]$ and the optical rotation of the solution in a 1 dm. tube are plotted against the total nitrogen content per milliliter of the suspension. The values corresponding to solutions obtained from the undersaturated side are represented by solid dots and those corresponding to solutions obtained from the supersaturated side are represented by circles. The solid lines drawn for the curves representing the pepsin per milliliter and the optical activity were calculated on the assumption that the ratio of activity or optical activity to

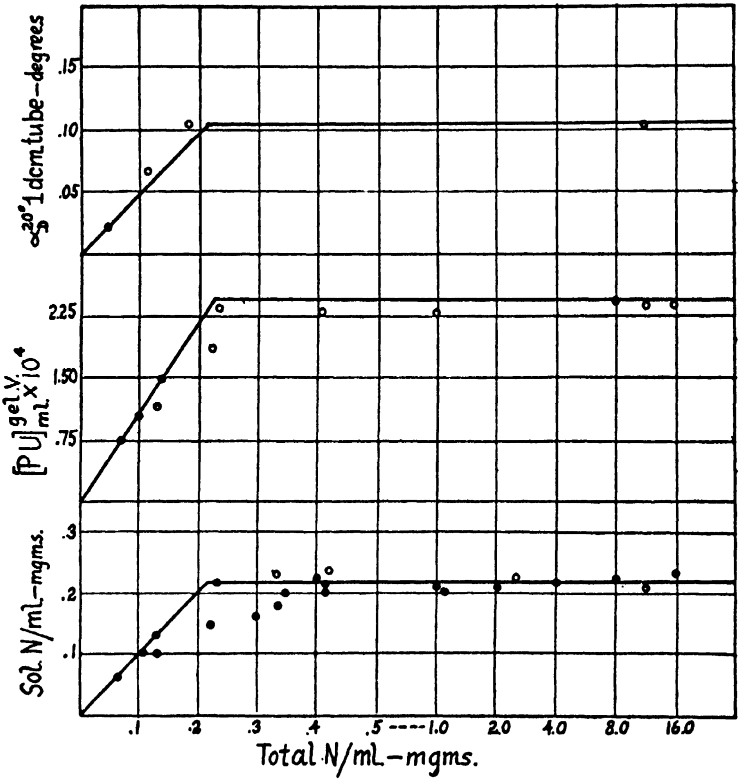


FIG. 2. Solubility of crystalline pepsin in 0,05 acetate and 0,5 saturated magnesium sulphate at 8°C.

nitrogen was the same in the solution as in the original crystals; that is, 1,15 proteolytic units per gram of nitrogen as measured by the liquefaction of gelatin, 92 proteolytic units per gram nitrogen as measured by the digestion of casein and a specific rotation per gram of nitrogen of −460° as measured at 22 to 24°C. with the sodium D line.

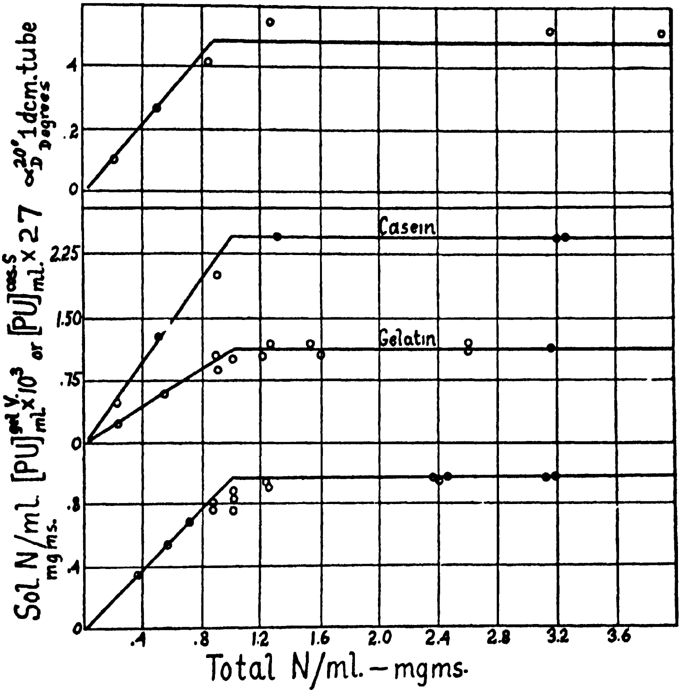


FIG. 3. Solubility of amorphous pepsin in 0,05 acetate and 0,5 saturated magnesium sulphate at 29°C.

The results show (1) that the solubility is independent of the quantity of solid present, (2) that the value for the solubility is an equilibrium value since it is the same whether obtained from supersaturated or undersaturated solutions, and (3) the specific proteolytic activity and the specific optical activity are the same for the material present in the solution as for the original material. The material behaves in regard to these determinations as a pure substance and there is no evidence of mixture. They show conclusively that the activity cannot be ascribed to the presence of a minute amount of a very highly active material mixed with the protein, unless it be further assumed either that this active material has about the same solubility as the protein or that it forms solid solutions with the protein. If the active material were soluble and mixed with the protein the activity per milliliter of solution would continue to increase as more and more solid material was added. While if it were present as a relatively insoluble material the activity per milliliter of the supernatant solution would become constant before the solubility of the protein had become constant. The figures show, however, that the proteolytic activity of the liquid becomes constant at the same point at which the concentration of the protein in the solution becomes constant. The only mixture which would behave in this way would be one in which the relative amounts of active material and protein were in almost exact proportion

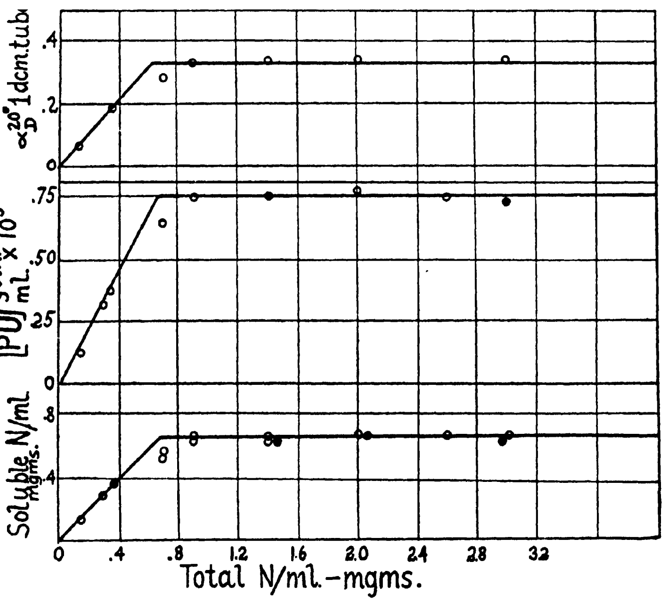


FIG. 4. Solubility of amorphous pepsin in 0,01 acetate and 0,5 saturated magnesium sulphate at 20°C.

to the relative solubilities of the two substances. This might conceivably be the case in one solvent but the possibility that this relation would hold in several solvents appears so small as to be negligible.

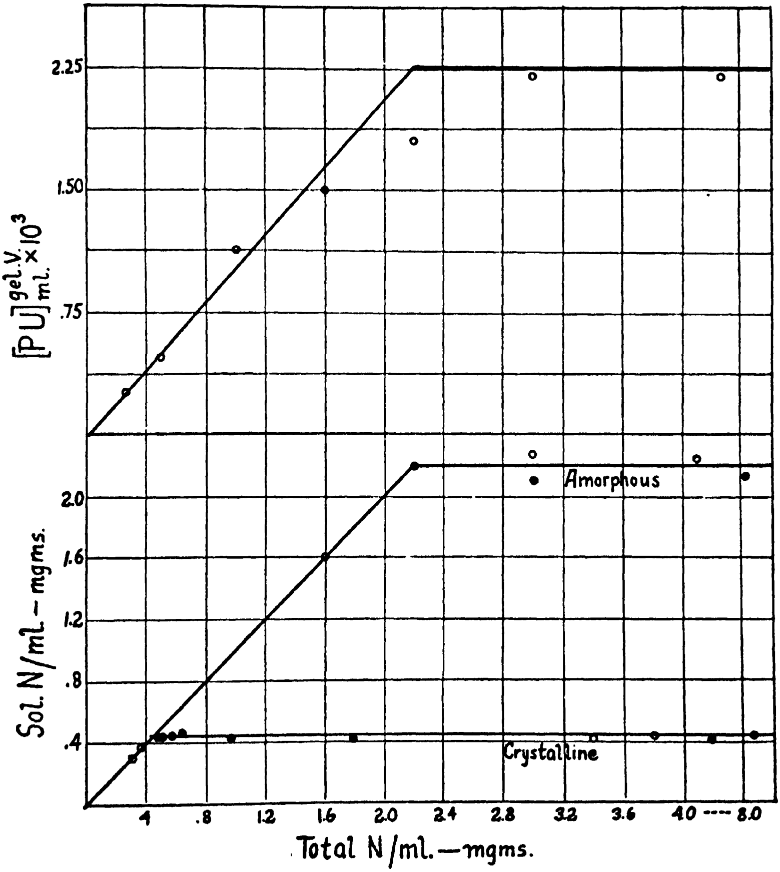


FIG. 5. Solubility of crystalline and amorphous pepsin in 0,055 acetate and 0,444 saturated magnesium sulphate at 20°C.

There remains only the possibility that the active material forms a solid solution with the protein and is either present in small amount or has about the same solubility as the protein. If it were present in small amount and dissolved in the protein, its presence would not affect the total solubility curve. The ratio of the active material to the protein in the precipitate at the point where the precipitate first appears would differ, however, from the ratio in the original material. The ratio of activity to protein in the solution in that part of the curve where there is a large amount of solid present would also differ from the ratio in the original material. The figures show that this latter condition does not occur. The activity per gram of nitrogen in the solution in equilibrium with a large excess of precipitate is the same within the experimental error as the ratio in the original material. The activity-nitrogen ratio of the first precipitate to appear is also the same in the experiments with amorphous and crystalline material as is shown in Table VI. If old preparations which have not been freed from the decomposition products are used, the activity-nitrogen ratio at this point will be low. An indication of this is shown in the first figure in the table and in some of the figures.

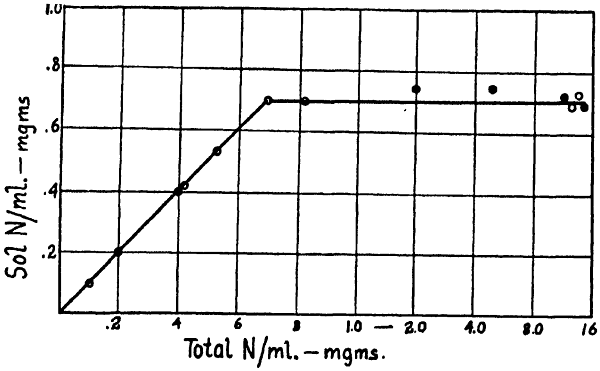


FIG. 6. Solubility of crystalline pepsin in 0,522 *M* sodium sulphate and $2{,}5 \cdot 10^{-5}$ *N* sulfuric acid at 20°C.

The material is therefore either a pure substance or a solid solution the components of which have nearly the same solubility in all the solvents tried.

A summary of the solubilities in the various solvents is given in Table VII.

c) *Inactivation.*—It was pointed out by Pekelharing that the temperature coefficient for the inactivation of enzymes and especially of pepsin was extremely high and agreed with that for the denaturization of proteins. No other reaction known has such an enormous temperature coefficient and the agreement of the two values suggests that the enzymes are proteins. Pekelharing also showed that loss of activity in strong acid solution was accompanied by the appearance of denatured, insoluble protein. It can be shown that inactivation either by heat or by alkali is quantitatively proportional to the denaturization of the protein.

TABLE VI

Ratio $\frac{Activity}{Nitrogen} = [PH] \frac{\text{gel. V.}}{\text{gm. N}}$

Experiment	Pepsin	Solvent	In precipitate when nearly all soluble	In solution when large excess of solid present	In original crystals
26	Crystals	0,05 acetate pH 4,65, 0,50 sat. $MgSO_4$	0,75	1,00	
			1,10	1,09	1,11±0,05
23	"	0,05 " " 4,65, 0,50 " "	1,2	1,11	
22	Amorphous	0,05 " " 4,65, 0,50 " "	1,20	1,16	
21	"	0,01 " " 4,65, 0,50 " "	1,09	1,02	
				1,13	
20	"	0,01 " " 4,65, 0,50 " "	1,11	1,21	

TABLE VII

Solubility of Pepsin

Preparation	Solvent	Temperature	Solubility N/ml.
			mg.
Crystals	0,522 M Na_2SO_4, 2,5 × 10^{-5} N H_2SO_4	20°	0,70
"	0,444 M Na_2SO_4, 0,0556 acetate buffer pH 4,65	20°	,43
Amorphous	0,444 M Na_2SO_4, 0,0556 acetate buffer pH 4,65	20°	2,2
Crystals	0,05 acetate pH 4,65, 0,50 sat. $MgSO_4$	8°	,22
Amorphous	0,05 acetate pH 4,65, 0,50 sat. $MgSO_4$	20°	1,00
"	0,01 acetate pH 4,65, 0,50 sat. $MgSO_4$	20°	,65

d) *Inactivation by Alkali.*—It is well known that pepsin is very sensitive to alkali. Goulding, Wasteneys and Borsook¹) showed that

---

¹) A. M. Goulding, H. Wasteneys and H. Borsook, J. Gen. Physiol. **10**, 451. 1926–27.

this inactivation could be separated into two reactions, one of which was instantaneous; and further that if the amount of this inactivation were plotted against the p_H a titration curve with a p_H of 6,85 was obtained. These experiments were repeated and the amount of inactivation compared with the amount of denatured protein found.

The results of such an experiment are shown in Fig. 7. The inactivation of the pepsin is quantitatively parallel with the denaturization of the protein since the percentage of activity left is the same as the percentage of soluble nitrogen remaining. The curve is very similar to that of Wasteneys and Borsook but is a little more to the alkaline side.

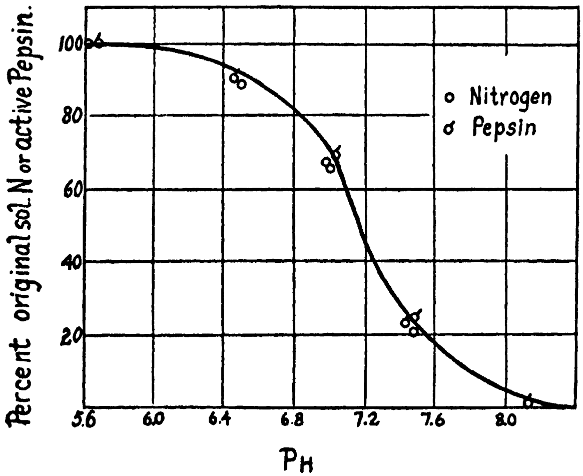


FIG. 7. Percentage inactivation and percentage denaturization of pepsin at various p_H at 20°C.

e) *Inactivation by Heat.*—Pekelharing noted that when pepsin solutions were allowed to stand in a range of acidity where the pepsin is active, the protein was digested and at the same time the activity of the solution decreased. He concluded from this that the pepsin digested itself. It can easily be shown, as was mentioned above, that the denatured protein is rapidly digested and it seems probable that the effect noticed by Pekelharing was the result of denaturization of the protein followed by digestion. It is then unnecessary to suppose that the enzyme digests itself. Pepsin solutions when heated slowly or when maintained at a temperature at which the inactivation proceeds slowly, do not contain denatured, insoluble protein since the latter is hydrolyzed as rapidly as it is formed. Under these conditions inactivation of pepsin is accompanied by the appearance of soluble nitrogen instead of insoluble; and it is difficult to make a sharp separation of the original pepsin from the products of hydrolysis. The result of an experiment which shows the digestion of denatured pepsin by active pepsin is given in Table VIII in which mixtures of various amounts of active and denatured pepsin were made up at p_H 3 and left at 35°C. for 24 hours. The denatured pepsin is precipitated by M/1 Na_2SO_4 at p_H 3, while the active pepsin is soluble. The table shows that the inactive and insoluble pepsin is dissolved by the active pepsin.

TABLE VIII

Digestion of Inactivated Pepsin

p_H 3,0, 35°C.
Pepsin dissolved in HCl, p_H 3.0 = Solution A
50 ml. A boiled, cooled = Solution D
A and D mixed in various proportions,
2 ml. sample of mixture taken and
2 ml. M/1 Na_2SO_4 added, filtered,
N/ml. determined in filtrate

A	20	10	5	0
D	0	10	15	20
Mg. soluble N in filtrate after:				
0 hrs.	,30	,11	,07	0
20 "	,30	,30	,28	,01

It may be shown, however, by means of solubility experiments that the loss of inactivation in the solution is exactly paralleled by the disappearance of the original protein. If a saturated solution of pepsin in M/300 hydrochloric acid is placed at 65°C. the activity is rapidly lost so that only about half the original activity remains after 15 minutes. No precipitate occurs but the formol titration increases. If the solution is now cooled and stirred again with a large quantity of crystalline pepsin more of the crystals must go into solution to replace the material which has been changed. If the inactivation of the enzyme is accompanied by some chemical change in the protein then the amount of material which will dissolve must be proportional to the percentage loss in activity. That is, if half the activity is lost the amount of protein which dissolves in this solution must be equal to half the original solubility (assuming the solubility to be unaffected by the presence of the inactivated enzyme). On the other hand, the activity per milliliter of the solution after being stirred with the solid should return to its original value. The result of an experiment designed to test this assumption is shown in Table IX. The solution was heated for 15 minutes at 65°C. and in that time the activity per milliliter had been reduced to 50 per cent of its original value, whereas the formol titration had increased 50 per cent and the total N per milliliter was unchanged since no precipitate appeared. The solution was then cooled and stirred at 20°C. with more crystals. The suspension was then filtered and the supernatant analyzed. The activity per milliliter is now the same as that of the original solution while the nitrogen per milliliter is 50 per cent higher than the original value. The formol titration has been increased by 50 per cent of its original value also. The results show that the loss of activity is exactly paralleled by some chemical change in the protein molecule.

TABLE IX

Inactivated Pepsin and Solubility. 65°C.

	$[PU]^{\text{gel. V.}}_{\text{ml.}\cdot 10^3}$	N/ml.	Formol/ml.
		mg.	*ml.* 0,01 NaOH
M/300 HCl, stirred 1 hr. with pepsin 20°C. Centrifuge	0,315	0,30	0,22
Supernatant heated at 65°C. for 15 min.	,160	,30	,33
Pepsin crystals added and stir 1 hr. Centrifuge			
Supernatant Observed	,325	,45	,45
Supernatant Calculated	,315	,455	,44

f) *The Rate of Inactivation at 65°C.*—It has frequently been found that the rate of inactivation of enzymes does not follow the course of a simple monomolecular reaction but becomes slower as the reaction proceeds. It was found by the writer[1]) that this peculiarity was connected with the purity of the enzyme solution and that the rate of inactivation of more highly purified solutions agreed with the monomolecular reaction rate, while those solutions containing inhibiting substances did not. On this basis the rate of inactivation of the crystalline material would be expected to be monomolecular. The result of an experiment in which the rate of inactivation of a 0,06 per cent solution of pepsin in 0,001 M hydrochloric acid (p_H 3) at 65°C. is shown in Fig. 8. The reaction follows the theoretical monomolecular course quite closely.

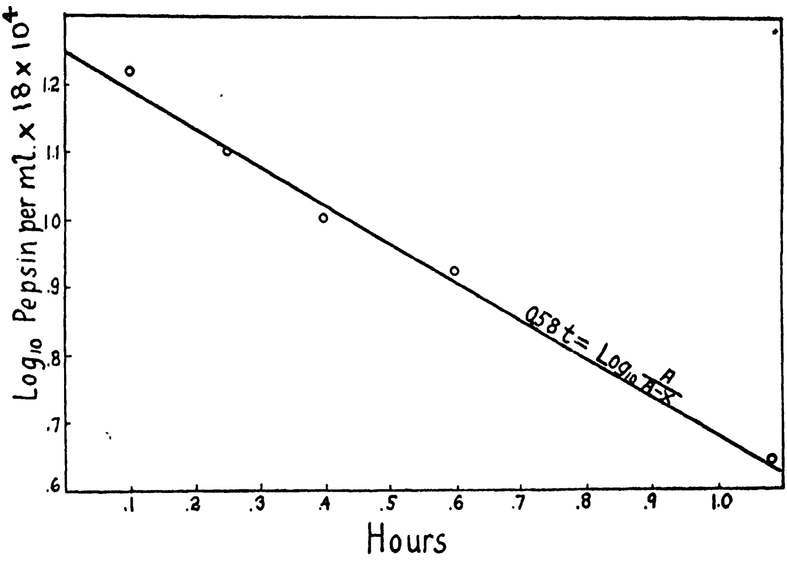


FIG. 8. Rate of inactivation of 0,06 per cent pepsin in 0,001 M hydrochloric acid at 65°C.

g) *Identity of the Diffusion Coefficient of the Protein and of the Active Material.*—The preceding experiments have shown that the activity cannot be separated from the protein molecule either by solubility measurements or inactivation experiments. It might be assumed, however, that the crystalline substance was analogous to a double salt and that the active material and the protein were in equilibrium with each other but were separate in solution. If this were the case it would be expected that the rate of diffusion of the two would be different. The diffusion coefficients were therefore determined by the method previously described1). The experiments were carried out at 8°C. and the amount of material diffusing determined by analyzing for nitrogen and also by activity determinations. The results of this experiment are shown in Table X. The diffusion coefficient was found to be the same when determined by either nitrogen or by activity. The radius (and presumably the weight) of the molecule containing the nitrogen and of the active molecule must therefore be the same, so that there is no indication that the protein molecule is different from the molecule responsible for the activity even in solution. The diffusion coefficient is found to be 0,047 ± 0,0005 cm^2/per day which, according to Einstein's formula, gives a molecular weight of 36000. This figure agrees very well with the molecular weight of 37000 as determined by osmotic pressure measurements. The agreement between these two figures shows that the molecules must be nearly all of the same size since the osmotic pressure gives a mean value while the diffusion experiment as carried out gives a minimum value. The agreement also shows that the molecule is not hydrated to any great extent since the value used for the specific gravity in calculating the molecular weight from the diffusion coefficient is the specific gravity of dry protein; and the value for the molecular weight so obtained agrees with that found by osmotic pressure.

---

[1]) J. H. Northrop, J. gen. Physiol. 4, 261. 1922.

TABLE X

Diffusion Coefficient, Radius of Molecule and Molecular Weight

8°C. 1 per cent solution in M/1 acetate buffer pH 4,6, viscosity of buffer = 0,017.
D from activity determination = ,047 ± ,0005 cm^2/day
from N determination = ,0468 ± ,0005 cm^2/day
= 5,44 × 10^{-7} cm^2/sec.

r = 2,22 × 10^{-7} cm.
= 2,22 mμ
M = 36000 ± 1000

---

1) J. H. Northrop and M. L. Anson, J. gen. Physiol. **12**, 543. 1929.

Preliminary experiments¹) gave larger diffusion coefficients corresponding to a molecular weight of about 10000 but this result was found to be due to an error in calibrating the diffusion cell.

h) *Recovery of Reactivated Pepsin, after Inactivation by Alkali.*—Pawlow and Parastschuk²) showed that pepsin, inactivated by alkali, regained some of its activity when the solution is partly neutralized. The conditions for this reactivation are strikingly similar to those used by Anson and Mirsky³) for the reversal of denaturization in other proteins. It was shown above that the inactivation of pepsin by alkali was quantitatively parallel to the denaturization of the protein. It could be assumed, however, that the enzyme was freed from the protein when the latter became denatured, and that the enzyme, itself, then became inactive. If the denatured protein could be changed back to the native state and if it then had the original proteolytic activity, very strong proof would be furnished that the activity was a property of the protein molecule itself. The only alternative would be to suppose that the conditions for reactivating the enzyme were the same as those for reversal of denaturization of the protein. This possibility appears so remote as to be negligible.

It was found possible⁴) to recover active pepsin from solutions which had been completely inactivated by alkali and this pepsin was indistinguishable from the original crystalline preparation. The yield is very poor but control experiments show that the active material recovered is many times greater than remained in the solution after inactivation, previous to the reactivation. It appears to the writer that this furnishes very strong proof of the identity of the enzyme and protein molecule.

i) *Antipepsin.*—If the enzyme is a protein an antibody should be formed on injection into an animal. The production of such antienzymes have been reported in the literature but the results are uncertain, as Euler has pointed out⁵), because of lack of information in

---

¹) J. H. Northrop, Science **69**, 580. 1929.

²) I. Pawlow and S. W. Parastschuk, Z. physiol. Ch. **42**, 415. 1904.

³) M. L. Anson and A. E. Mirsky, J. gen. Physiol. **9**, 169. 1925.

⁴) J. H. Northrop, J. gen. Physiol. **14**, 713. 1931.

⁵) H. Euler, Chemie der Enzyme, 3rd edition, Part 1, p. 390, Bergmann, München 1925.

regard to the activity of the preparation used. In order to see if any antibody could be produced and if its production were associated with the activity of the preparation, two rabbits were immunized, one with an active preparation of the enzyme and one with a preparation of the same concentration which had been inactivated by alkali. 5 ml. injections of a 1 per cent solution of the active or inactive material were made intraperitoneally at intervals of about 1 week. Four injections were made; the rabbits were bled 2 weeks after the final injection and the serum obtained in the usual way. These sera gave a precipitin reaction with the pepsin solution, as shown in Table XI. Normal serum gave no precipitate with either the active or the inactive pepsin preparation. The serum obtained from the rabbit injected with active pepsin gave a positive precipitin reaction in a dilution of about 1/2000 with the active pepsin solution and to about 1/16 with the inactive solution. The serum from the rabbit immunized with denatured pepsin gave a precipitin test with the denatured preparation in a dilution of about 1/8 per cent solution and with the active preparation to about the same extent. Both sera gave precipitin reactions with a 1 per cent solution of pepsin inactivated by boiling in a dilution of about 1/16. As might be expected, therefore, the sera were not strictly specific but gave cross precipitations with both active and denatured pepsin. The inhibiting effect of the sera on the activity was tested by mixing increasing amounts of the antiserum with a small amount of active

TABLE XI

Antiserum

1 ml. 1 per cent pepsin solution in M/5 4,6 acetate buffer, diluted with buffer, + 0,15 ml. serum

Pepsin dilution		1	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$	$\frac{1}{1000}$	$\frac{1}{2000}$	0
Pepsin	Serum	O serum	,15 ml. serum												
Active	Active	−	++	++	++	++	++	+	+	+	+	+	+	+	−
"	Denatured	−	++	++	++	++	+	−	−	−	−	−	−	−	−
"	Normal	−	−	−	−	−	−	−	−						
Denatured	Active	−	++	++	+	+	+	−	−	−	−	−	−	−	−
"	Denatured	−	++	++	++	+	−	−	−	−	−	−	−	−	−
"	Normal	−	−	−	−	−	−	−	−	−	−	−	−	−	−

pepsin and determining the rate of digestion of casein or gelatin with this mixture. The result of this experiment is shown in Table XII.

Both the serum prepared by the injection of the active pepsin and that prepared by the injection of the denatured pepsin inhibit the action of the enzyme to about the same extent when tested by the solution of casein (cas. S. method). The normal serum inactivates somewhat less although there is no very great difference. It is difficult to compare the action of the various sera quantitatively from this experiment. The effect may be seen more clearly by determining the rate of hydrolysis of gelatin (gel. V. method) with a small amount of pepsin to which has been added increasing amounts of the various sera. The result of such an experiment is also shown in Table XII. The active serum inhibits quite strongly in a dilution of 1:4, the denatured serum less so and the normal serum still less. This inhibiting effect is evidently very weak compared with that of many antitoxins, although the precipitin reaction is about what would be expected. This may be due to the fact that the pepsin injected must be almost instantly inactivated since, as was shown above, the pepsin

TABLE XII

Effect of Antiserum on Digestion of Casein or Gelatin by Pepsin

Serum diluted as noted + M/5 4,6 acetate buffer. Pepsin in 4,6 acetate buffer
1 ml. pepsin solution + 1 ml. serum dilution

Gelatin

	Final dilution of serum					
Serum	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	0
	$[PU]^{\text{gel. V.}}_{\text{ml.}}$ after 3 hrs. $\cdot 10^3$					
Normal	0,075	0,10	0,11	0,12	0,11	0,12
Antidenatured	,060	,10	,09	,10	,11	,12
Antiactive	,045	,075	,10	,10	,11	,12

Casein

Serum	0	Normal	Anti-denatured	Antiactive
Soluble N in filtrate, mg	2,2	2,0	1,87	1,87
$\asymp [PU]^{\text{cas. S.}}_{\text{ml.}}$	,015	,008	,007	,007

is instantly more than half inactivated at a p_H of 6. It is probable therefore that the active pepsin is in the circulation of the animal for only a very short time.

The writer is indebted to Dr. F. S. Jones and Dr. A. P. Krueger for advice and assistance in connection with these experiments.

6. Conclusions as to the Purity of the Preparation

The preceding experiments have shown that no evidence for the existence of a mixture of active and inactive material in the crystals could be obtained by recrystallization, solubility determinations in a series of solvents, inactivation by either heat or alkali, or by the rate of diffusion. It is reasonable to conclude therefore that the material is either a pure substance or a solid solution of two very closely related substances. If it is a solid solution of two or more substances it must be further assumed that these substances have about the same degree of solubility in the various solvents used, as well as the same diffusion coefficient and rate of inactivation or denaturization by heat. It must also be assumed that both substances are changed by alkali at the same rate and to the same extent, and further that the conditions for reactivating the enzyme after alkali inactivation, are the same as those for reversing the denaturization of the protein. This could hardly be true with the possible exception of two closely related proteins. It is conceivable that two proteins might be indistinguishable by any of the tests applied in this work. But in this case it would follow that the enzyme itself was a protein and this, after all, is the main point. It does not necessarily follow even if the material represents the pure enzyme that it is the most active preparation that can be obtained nor that it is the only compound which has proteolytic activity. It is possible that hemoglobin is the type structure for the enzymes and that they consist of an active group combined with a protein as suggested by Pekelharing. The active group may be too unstable to exist alone, but it is quite conceivable that a series of compounds may exist containing varying numbers of active groups combined with the protein, and that the activity of the compound would depend on the number of these active groups. This hypothetical complex would not differ much from that assumed by Willstätter¹) and his coworkers,

---

¹) R. Willstätter, Naturw. **15**, 585. 1927.

TABLE XIII

Summary of Properties of Pepsin

Crystalline form. Dihexagonal bipyramids, colorless, 0,01 to 0,1 mm. long. Positive double refraction.
Density. 1,32 gm. per ml.

$[\alpha]_D^{24°}$ (pH 4–5) – 70°

Analysis. Per cent of dry weight.	Total N	Amino N	C	H	Cl	S	P	Ash	$SO_4^=$
	15,3	,80	52,4	6,67	,22	,86	,078	,40	00

Xanthoproteic and Biuret test — positive
Molisch test — negative

Diffusion coefficient 8°		
	M/1 acetate buffer pH 4,6 Viscosity ,017	,047 ± ,0005 cm²/day (From N determination) ,0468 ± ,0005 cm²/day (From activity determination)

Molecular weight from osmotic pressure	
(M/1 acetate pH 4,6)	35 000 ± 1000
From diffusion coefficient	36 000 ± 1000
From P content, 1 atom P per mole	40 000
C " 2 atoms Cl per mole	35 000
S " 10 " S " "	36 000

Solubility	Solvent	,002 HCl	,522 *M* Na_2SO_4 2,5 × 11^{-5} *N* H_2SO_4	,444 *M* Na_2SO_4 ,055 acetate pH 4,65	,05 sat. $MgSO_4$ 0,5 acetate pH 4,65	0,5 sat. $MgSO_4$ 0,1 acetate pH 4,65
Mg. N/ml.	Crystalline	,3 (20°)	,70 (20°)	,43 (20°)	,22 (8°)	
	Amorphous			2,2 (20°)	1,00 (20°)	,65

Velocity constant inactivation
65° – pH 3,0 0,58 Time in hrs. Log_{10}.
1,98 per cent solution in HCl

Activity		Equivalents per mole pepsin per min. 35,5°C.
Substrate	*p*H	
Edestin	2,0	1000
Casein	2,0	500
Denatured egg albumin	2,2	320
Gelatin	2,5	6,0

except that it supposes a definite chemical compound of the active compound with the protective group, in place of an adsorption complex. It is of course possible that both types of complex may be formed under suitable conditions. The fact that the crystalline urease prepared by Sumner is also a protein and that the temperature coefficient for the rate of inactivation of enzymes in general is that characteristic for the denaturization of proteins, suggests that the protein fraction in the purification of enzymes be given special attention. A crystalline protein possessing constant, tryptic activity has recently been isolated by Kunitz and the writer¹).

The crystalline pepsin resembles the amorphous preparations obtained previously by Pekelharing, Ringer, Fenger, Andrews and Ralston and other workers. It is probable that the preparations obtained by these workers were nearly pure pepsin. Both Pekelharing and Ringer²) obtained preparations free from phosphorus so that there may be several proteolytically active forms.

Pekelharing showed that the same protein material could be obtained from gastric juice as well as from autolyzed gastric mucosa so that it is probable that the crystalline material could also be readily prepared from gastric juice. It seems fair to conclude therefore that the crystalline protein described in this paper is identical with the enzyme pepsin as secreted by the animal.

7. *Proteolysis Is a Homogeneous Reaction*

Sorensen has shown that a solution of egg albumin is a one-phase system and the solubility measurements described in this paper show that a solution of pepsin consists also of only one phase. The reaction between dissolved egg albumin and dissolved pepsin therefore is a case of homogeneous, rather than heterogeneous catalysis.

8. SUMMARY

A method is described for the preparation of a crystalline protein from commercial pepsin preparations which has powerful peptic activity. The composition, optical activity, and proteolytic activity of this protein remain constant through seven successive crystalliza-

---

¹) J. H. Northrop and M. Kunitz, Science **73**, 262. 1931.

²) W. E. Ringer, Z. physiol. Ch. **95**, 195. 1915.

tions. No evidence for the presence of a mixture or of a solid solution is found in a study of the solubility of the protein in a series of different salt solutions, or from the diffusion coefficient or rate of inactivation. These results indicate that the material is a pure substance or possibly a solid solution of two or more substances having nearly the same solubility in all the various solvents studied. It seems reasonable to conclude from these experiments that the possibility of a mixture must be limited to a mixture of proteins, so that the conclusion seems justified that pepsin itself is a protein. The isoelectric point of the protein is at p_H 2,85. A titration curve has been determined. A number of other properties have been determined, and may be summarized as follows: Table XIII (S. 223).

[Reprinted from The Journal of General Physiology, September 20, 1932,
Vol. 16, No. 1, pp. 33–40]

CRYSTALLINE PEPSIN

IV. Hydrolysis and Inactivation by Acid

By JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, May 20, 1932)

Experiments with crystalline pepsin already described (1–3) show that the crystalline protein has a constant and characteristic proteolytic activity. The proteolytic activity of various preparations is the same and cannot be changed by repeated recrystallization, partial solution in various salt concentrations, or by fractional diffusion. The loss of activity when the protein is denatured by alkali corresponds to the amount of denatured protein formed and when this denatured protein is changed back to native protein the original activity is regained. These results render it very improbable that the activity is due to the presence of any non-protein molecule adsorbed on the protein molecule since adsorption complexes in general do not have constant properties. They indicate that the proteolytic activity is a property of this molecular species of protein, just as the peculiar properties of hemoglobin are attributes of the molecule of the protein, hemoglobin. In both cases the characteristic property is lost when the protein is denatured and regained when the protein is again brought into the native form (2, 4). In the case of hemoglobin it is known that the characteristic properties are due to the presence of a special prosthetic group in the molecule and it is probable that the pepsin protein also contains some characteristic group to which its activity is due. There is at present no evidence as to the nature or even the existence of such a characteristic group and, owing to the difficulty of determining the structure in the case of proteins, it is difficult to obtain such evidence by the ordinary methods of structural chemistry. The problem would be greatly simplified if a part of the protein molecule possessing even very slight activity could be split off. The attempt was therefore made to hydrolyze pepsin under conditions which would not completely destroy the activity and to determine whether a solution could be obtained which was more active than would be expected from its content of protein. If any of the split products of the protein were appreciably active compared to the original protein molecule the total activity of the solution would be higher than that calculated from its protein content.

Unfortunately the methods of hydrolyzing the protein are limited practically to dilute acid. Several attempts were made to hydrolyze pepsin with trypsin at pH 6 which is as far on the alkaline side as it is possible to work without causing alkali inactivation. The trypsin is, however, very rapidly destroyed by the pepsin and no hydrolysis of the pepsin could be detected so that there was no change in either the protein concentration or the activity when trypsin was added to pepsin solutions. Attempts to hydrolyze the protein with papain were also unsuccessful. No hydrolysis of the pepsin protein or decrease in peptic activity could be detected. Most of the protein present in the papain preparation used was very rapidly digested and there was a slight loss in papain activity so that the results indicate that papain is digested by pepsin although very much more slowly than is trypsin.

Hydrolysis in Dilute Acid

It is known that pepsin solutions lose their activity slowly when allowed to stand in dilute acid and it was shown in the first paper (1) that this loss in activity was exactly equivalent to the amount of native protein destroyed, since if a saturated solution of the protein crystals is partially inactivated and then saturated again with the crystals, the activity of the solution returns exactly to its original value while the total nitrogen content increases to just the extent calculated from this increase in activity. This is a very sensitive method since the solubility is probably the most specific property of a protein. It cannot be used, however, to follow the hydrolysis very far since the products formed by the hydrolysis, when present in considerable quantities, begin to affect the solubility of the crystals. In the present experiments, therefore, the concentration of protein nitrogen in the solution was determined directly by precipitating the protein with 2.5 per cent trichloracetic acid and also by determining the quantity of protein which becomes denatured and insoluble when the solution is made alkaline. This effect of alkali is a characteristic property of the pepsin protein and it seemed possible that the figure obtained in this way might differ from that obtained with 2.5 per cent trichloracetic acid since the latter reagent might precipitate other substances than the unchanged protein itself. However, the results showed that exactly the same amount of protein nitrogen was determined by both methods. In addition, the amount of nitrogen precipitated by phosphotungstic acid was also determined. The loss in activity was determined by several methods.

TABLE I

Decrease in Activity and Protein Nitrogen in Pepsin Solutions at Different pH

5 gm. three times crystallized pepsin stirred with 50 ml. H_2O and dissolved by addition of M/5 NaOH. 5 ml. of this solution added to 20 ml. various concentrations HCl; solution kept at 35°C. and analyzed for protein nitrogen (2.5 per cent CCl_3COOH) and activity.

Concentration HCl mols/*l*..	0.01			0.05			0.20			1.0			2.0		
Approximate pH.........	4.0			1.5			0.7			0			+0.3		
Hrs. at 35°C.	P.N./ml. mg.	$[P.U.]_{ml.}$		P.N./ml. mg.	$[P.U.]_{ml.}$		P.N./ml. mg.	$[P.U.]_{ml.}$		P.N./ml. mg.	$[P.U.]_{ml.}$		P.N./ml. mg.	$[P.U\ ]_{ml.}$	
		Gel. V^-	Ed. V^-		Gel. V^-	Ed. V^-		Gel. V^-	Ed. V^-		Gel. V^-	Ed. V^-		Gel. V^-	Ed. V^-
0	0.80	10	780	0.82	11	770	0.81	10	800	0.80	12	790	0.80	12	785
24	0.80	9	770	0.52	8	620	0.43	7	500	0.25	4	280	0.11	1	100

Effect of pH

As a preliminary experiment a series of solutions of crystalline pepsin containing different concentrations of hydrochloric acid were kept at 35°C. for 24 hours and the protein nitrogen per ml. of solution precipitated with 2.5 per cent trichloracetic acid was determined at the beginning and the end of the experiment. The results of this experiment are shown in Table I. The table shows that the more acid the solution the more rapidly the protein is hydrolyzed so that with 2 molar hydrochloric acid only about 10 per cent of the original protein is present after 24 hours. The table also shows that the decrease in activity is proportional to the decrease in protein nitrogen. The effect of pH on the stability of the protein therefore is similar to that already found for crude pepsin preparations (5).

Mechanism of the Reaction

There are several possible mechanisms which could account for the hydrolysis of the protein under these conditions.

1. The reaction is analogous to ordinary acid hydrolysis of proteins and differs only in that it proceeds more rapidly than is the case with other proteins.
2. The first step in the reaction is the formation of denatured protein which is then hydrolyzed by the acid.
3. The protein digests itself, or denatured protein is formed, by the acid and this denatured protein is then digested by the remaining active protein.

The results as a whole indicate that the reaction is simply a case of ordinary acid hydrolysis and that no denatured protein is formed. The denatured protein is completely insoluble in solutions more acid than pH 3 and if such solutions are heated to boiling rapidly the protein is completely precipitated. No precipitate appears in any of the solutions in the preceding experiment and therefore no appreciable amount of denatured protein is present. In general, there is an optimum pH for the digestion of proteins by pepsin, while the denaturation of proteins increases with the concentration of acid and no optimum is obtained. If, therefore, the first step in the reaction were the formation of denatured protein by the acid and this denatured protein were subsequently hydrolyzed by the active enzyme, it would be expected that in very strong acid solution denatured protein would accumulate in the solution since there should be a point on the acidity curve where the rate of digestion would be decreased by increasing acid while the formation of denatured protein would increase. This is not the case and indicates quite strongly that the observed hydrolysis is not due to enzyme activity. The evidence is not so clear in regard to the possibility that denatured protein is the first step in acid hydrolysis itself. It would be expected that the rate of hydrolysis of denatured protein would increase with the acidity just as does the formation of denatured protein. If it is assumed, therefore, that the denatured protein is more rapidly hydrolyzed than the native protein, then there would be no accumulation of denatured protein at any degree of acidity and the results would agree with this mechanism. As a matter of fact, if the protein is denatured by boiling and then kept at 50°C. it is found to hydrolyze less rapidly than a similar solution which has not been boiled. The boiled protein, however, is present in the form of a precipitate and it could be supposed that this was the reason for the slow rate of hydrolysis. On the other hand, there is no evidence for the existence of any denatured protein in any of the solutions at any time so that the assumption that the first step in the reaction consists in denaturation is hardly justified. For the time being, therefore, the reaction will be considered simply as a case of ordinary acid hydrolysis.

Decrease in Protein Nitrogen and Activity at pH 1.8

If any of the products of the hydrolysis of the pepsin were active their presence might be shown by changes in the relative velocity with which the solution hydrolyzes different proteins as well as by a change in the specific activity of the protein in the solution with regard to one substrate. In order to determine whether such changes, either in specific activity or comparative activity with different proteins, occur during the hydrolysis of the pepsin an experiment was carried out at about pH 1.8 and at 50°C. in which the decrease in protein nitrogen and the decrease in activity were determined. The activity was measured by the change in viscosity of gelatin, casein, or edestin, the rennet action on milk, the formation of non-protein nitrogen from casein and edestin, the rate of increase in formol titration of edestin and gelatin, and also the digestion of hemoglobin.

About 2 gm. of four times recrystallized pepsin was stirred into 50 ml. of water and dissolved by the careful addition of 1 ml. M/1 sodium hydroxide. 10 ml. of M/1 hydrochloric acid was then added rapidly and the solution placed at 50°C. 10 ml. samples were withdrawn at various time intervals and 2 ml. M/1 sodium acetate added in order to bring the pH to about 4. The samples were stored in the ice box until the experiment was completed and then analyzed for protein nitrogen and for activity by the various methods mentioned above.

The results of the experiment are shown in Table II. The table shows that the protein nitrogen, as determined by either alkali denaturation or precipitation with 2.5 per cent trichloracetic acid decreases from 1.9 mg. per ml., its original value, to 0.06 while the protein nitrogen, as determined by precipitation with phosphotungstic acid decreases only to about 0.5 mg. per ml. The activity of the solution as measured by any of the methods decreases in almost exact proportion to the decrease in the concentration of protein nitrogen as determined by 2.5 per cent trichloracetic acid. This fact may be best shown by expressing the results in terms of the specific activity per milligram protein nitrogen. This figure is obtained by dividing the observed activity per ml. of solution by the number of milligrams of protein nitrogen per ml. of solution. The results of the experiment calculated in this way are shown in Table II. The table shows that

TABLE II

Hydrolysis of Pepsin at 50°C. and pH 1.8

Sample	Protein nitrogen per ml.			$[\text{P.U.}]_{\text{mg. P.N.}}$ (Specific activity of protein N)											
	Alk.	2.5 CCl_3COOH	Ph. *T*	Gel. V^-	Cas.' V^-	Ed. V^-	Cas. V^+	Rent.	Cas. *S*	Ed. *S*	Cas. *F*	Ed. *F.*	Gel. *F*	Hb
1	1.90	1.80	1.90	13	1000	880	460	73×10^4	0.47	0.42	0.16	0.08	0.002	0.18
3	0.62	0.60	0.90	13.7	1050	870	350	69×10^4	0.42	0.39	0.17	0.08		0.17
5	0.20	0.21	0.50	13.0	1020	880	350	71×10^4	0.46	0.42	0.17	0.085	0.0025	0.18
6	0.06	0.06	0.45	18.0	980	890	420	71×10^5	0.38	0.46				0.18

the specific activity, as measured by any of the eleven methods used remains constant, within the limits of error of the method, throughout the entire experiment. They prove, therefore, that the activity is entirely due to the original protein remaining in solution and that if any of the split products of the protein possess any activity it must be less than 5 per cent of that possessed by the protein molecule itself. The methods are not sufficiently accurate to detect changes in activity of less than 5 per cent and it is still possible that some of the split products may have a very low activity. In addition to the constant activity the figures also show that there is no change in the relative activity as determined with different proteins. The experiment cannot be continued further since five hundredths of a milligram protein nitrogen per ml. approaches the limit of accuracy of protein nitrogen determinations although the activity measurements are very much more sensitive. It is necessary, however, to be able to compare the activity with the protein nitrogen content and no information can be obtained from activity measurements made on a solution containing too little protein nitrogen to determine quantitatively.

EXPERIMENTAL METHODS

Activity Measurements were made as already described (6).

Protein Nitrogen Methods

2.5 Per Cent Trichloracetic Acid.—1 ml. of solution is added to 10 ml. of 2.5 trichloracetic acid, heated to 80°C. for 10 minutes, and then cooled. The precipitate is centrifuged, washed three times with 2.5 trichloracetic acid, dissolved in dilute sodium hydroxide. The solution is washed into the Kjeldahl flask and the total nitrogen determined.

Alkali Protein.—2 ml. of the solution is added to 2 ml. M/5 sodium hydroxide, 2 ml. N/5 hydrochloric acid added and then 5 ml. of a solution of M/2 sodium sulfate and M/200 sulfuric acid. The suspension is centrifuged, washed three times with the sodium sulfate solution, the precipitate dissolved in dilute sodium hydroxide, and the total nitrogen content determined by the Kjeldahl method.

Phosphotungstic Acid.—Reagent—1.5 gm. of phosphotungstic acid dissolved in 5 ml. concentrated hydrochloric acid, made up to 100 ml. with ethyl alcohol, and then made up to 500 ml. with water. 1 ml. of the solution is added to 5 ml. of this reagent, the suspension centrifuged, washed three times with the reagent, the precipitate dissolved in dilute sodium hydroxide and the total nitrogen content determined by the Kjeldahl method.

SUMMARY

The decrease in protein nitrogen and in the activity of solutions of crystalline pepsin at pH 1.8 and 45°C. has been determined. The decrease in activity, as measured with eleven different methods, is in exact proportion to the decrease of protein nitrogen of the solution. The measurements were continued until less than 5 per cent of the original protein remained. These results indicate that none of the split products of the protein molecule possess any appreciable activity compared to that of the original protein.

BIBLIOGRAPHY

1. Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.
2. Northrop, J. H., *J. Gen. Physiol.*, 1931, **14**, 713.

3. Northrop, J. H., *Ergebn. Enzymforsch.*, 1932, **1**, 302.
4. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, 1929, **13**, 133.
5. Northrop, J. H., *J. Gen. Physiol.*, 1920, **5**, 465.
6. Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 41.

[Reprinted from The Journal of General Physiology, September 20, 1932,
Vol. 16, No. 1, pp. 41–58]

PEPSIN ACTIVITY UNITS AND METHODS FOR DETERMINING PEPTIC ACTIVITY

By JOHN H. NORTHROP

(*From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Accepted for publication, May 20, 1932)

Catalysts, by definition, affect only the velocity of a reaction and the activity of a catalyst, therefore, should be expressed as a velocity constant. Since enzymes are generally considered to be catalysts the unit of enzyme activity should also be expressed as a velocity constant. In order to do this, however, it is necessary to know the kinetic equation for the reaction. In the case of most enzyme reactions this equation is not known and it is therefore not possible to express the activity of the enzyme as a velocity constant. This is especially true of pepsin and trypsin. It is necessary, therefore, to use some more or less arbitrary definition of activity and it has been found convenient in the case of pepsin to express the activity in terms of the initial rate of the reaction due to the presence of the enzyme. This definition of activity is useful for analytical purposes and may be used to compare the activity of various quantities of the same enzyme with the same substrate but cannot be used to compare the activity of different enzymes acting on different substrates.

Proteolytic activity, as defined in this way, corresponds to the measurement of optical activity and refers to a property of the solution. The activity caused by unit quantity of a preparation is called the specific activity and in this paper is expressed as units of activity observed per mg. protein-nitrogen added in the enzyme preparation. The specific activity is abbreviated as $[\text{P.U.}]^{\text{Prot.}}_{\text{mg.N}}$. In general the units are similar to those used by Euler (1) and Willstätter (2).

Selection of Methods

The methods described in the present paper have been developed in connection with the study of the purification of pepsin and have been chosen from a large number of possible methods with regard to their accuracy, convenience, and significance. When a protein solution is acted upon by pepsin a number of chemical and physical changes occur and it cannot be told beforehand whether or not these different changes are due to different enzymes. It is advisable, therefore, to use more than one change in the properties of the protein solution so as to detect the possible presence of more than one enzyme. For the same reason it is advisable to determine the activity with more than one protein. It was found, for instance, in the progress of the purification of pepsin that one fraction was extremely active in the liquefaction of gelatin (3), whereas it was not especially active in changing its chemical properties. The presence of this specific enzyme would not have been suspected had not the determinations been made in several different ways.

The first effect of pepsin on protein solutions is a very rapid decrease in viscosity accompanied by a slower decrease in the quantity of nitrogen present in the form of protein and by a still slower increase in the number of titratable carboxyl (or amino) groups. The optical activity and conductivity also change but the three former properties have been selected as characteristic of the various changes occurring during the digestion of a protein solution.

The method of determining the activity by the change in viscosity has the advantage of rapidity and accuracy (4). Four or five determinations may be completed in less than an hour and comparative determinations may be made with a probable error of less than 5 per cent. When the determination is made at different times with solutions prepared from various samples of proteins the variation is quite marked and may be 100 per cent owing to the difficulty of preparing protein solutions of the same properties at different times with different protein samples. Gelatin preparations are, however, quite reproducible and the change in the viscosity of gelatin has been found to be one of the most accurate, convenient, and reproducible methods. The change in viscosity has the disadvantage that there is some question as to its physical significance and especially as to its relation to the chemical changes (5, 6).

The method of determining the formation of non-protein nitrogen (*i.e.*, nitrogen not precipitated by 10 per cent trichloracetic acid) is more troublesome than the viscosity method. It has the further disadvantage that the quantity of non-protein nitrogen produced has no simple relation to the quantity of enzyme except when very small amounts of enzyme are used and under these conditions the determination is not very accurate. Like the viscosity method, the formation of non-protein nitrogen gives results which cannot be directly interpreted in terms of the chemical changes.

The method of determining the digestion by the increase in carboxyl (or amino) groups is much better in this latter respect since the chemical significance of this measurement is clear. The method is not especially accurate or convenient and the titration has to be carried out with considerable care owing to the fact that the increase in carboxyl groups is a very small per cent of the total titration of the solution. As in the case of the formation of soluble nitrogen, the increase in the formol titration is not proportional to the quantity of enzyme except with very small amounts of enzyme which again yield very small and uncertain titrations.

Selection of Proteins

Gelatin, edestin, casein, and a standardized solution of dried milk have been selected for the activity tests since these solutions represent widely different classes of proteins and are the most easily obtainable in preparations of more or less constant properties. In addition to these methods a colorimetric method for determining the formation of soluble tryptophane and tyrosine from hemoglobin has been developed by Anson (7).

Activity Units

An ideal unit of activity should contain factors for all the various conditions affecting the activity of the enzyme so that a constant figure for the activity would be obtained no matter how the determination was carried out. There are so many factors affecting the rate of peptic digestion, however, that it is not possible to collect them all into one constant factor and it is necessary to select arbitrary standard conditions for the determination. In fixing these conditions it is necessary to consider the constancy with which they can be reproduced and the danger of accidental variations, such as changes in the pH, etc. After these conditions have been selected it is again necessary to make an arbitrary selection of the units in which the activity is to be expressed. Since changes in different properties of the protein solutions are measured by various methods, it is necessary to use different dimensions for the activity units for the separate methods. The following definitions have been selected.

Unit of Peptic Activity as Defined by Changes in Viscosity of Gelatin, Casein or Edestin Solutions $[P.U.]^{\text{Protein } V}$.—One unit of activity is defined as a change in the specific viscosity of the standard protein solution at 35.5°C. at the rate of 1 per cent per minute, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the viscosity when contained in 5.2 ml. of the standard digestion mixture. This value is determined from the initial slope of the viscosity-time curve as described in detail below. The activity is expressed as per cent change since this value is nearly independent of the protein concentration. The temperature is fixed at 35.5°C. since at this temperature the viscosity of gelatin remains constant with time while at higher temperatures it decreases and at lower temperatures it increases.

Coagulation of Milk $[P.U.]^{\text{Ren.}}$.—In the case of the coagulation of milk the viscosity time curve is not straight but curves upward rapidly so that the initial slope cannot be determined as is the case with the preceding determinations. The percentage increase in the viscosity is therefore determined arbitrarily from the point at which the specific viscosity is doubled and is obtained by dividing 100 by the time in minutes required to cause this change. The physical significance of this figure is evidently the percentage increase in the viscosity per minute which would be obtained if the increase in viscosity with time were linear over this range.

Activity Units As Determined by the Formation of Soluble Nitrogen $[P.U.]^{\text{Prot. S}}$.—One unit of activity is defined as the liberation of non-protein nitrogen at the rate of 1 milliequivalent per minute at 35.5°C. in 6 ml. of standard digestion mixture, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of non-protein nitrogen when contained in 6.0 ml. of the standard digestion mixture. In this case actual rather than per cent change is used since the former is more nearly independent of the protein concentration. The figure may be obtained by interpolating from a series of determinations made with small amounts of enzyme at various times or from the initial slope of a curve obtained by plotting the change caused by increasing quantities of enzyme after the same time interval.

Activity Units As Defined by the Increase in Formol Titration $[P.U.]^{\text{Prot. F}}$.—One unit of activity is defined as an increase in carboxyl (or amino) groups in 6.0 ml. of the standard digestion mixture at 35.5°C. at the rate of 1 milliequivalent per minute, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of carboxyl groups when contained in 6.0 ml. of the standard digestion mixture. This figure is obtained in the same way as that for the increase in soluble nitrogen. It represents either the increase in carboxyl groups or the increase in amino groups, depending upon whether or not the zwitter ion theory of proteins and amino acids is correct. According to the classical theory it measures the carboxyl (acid) groups.

EXPERIMENTAL PROCEDURE

I. Viscosity

Standard Protein Solution

Gelatin.—12.5 gm. air-dry isoelectric gelatin prepared as described by Northrop and Kunitz (8) is stirred into 200 ml. of water; the suspension warmed to 45°C. and made up to 500 ml. A few crystals of thymol are added and the solution kept in the ice box. The relative viscosity of this solution should be between 2.1 and 2.3 at 35.5°C. The solution may be kept without change for several weeks at 5°C. but the viscosity decreases slowly at 35.5°C. so that only sufficient of the solution is warmed to 35.5°C. for 1 day's use. Isoelectric gelatin is used rather than acid gelatin since the viscosity of acid gelatin changes quite rapidly. Also the viscosity of isoelectric gelatin solutions is more easily reproducible and less sensitive to slight changes in pH and especially to the addition of small amounts of salts. Very much larger quantities of enzyme than would be required at pH 2 are needed and this also is an advantage since it obviates the necessity of making very high dilutions of the enzyme solution used.

Casein Solution.—25 gm. of Kahlbaum's casein (according to Hammarsten) are stirred into 500 ml. of water at about 40°C., care being taken to avoid the formation of large lumps. 25 ml. of molar hydrochloric acid are then added slowly and the solution stirred until the casein is all dissolved. The flask is then placed in boiling water for ½ hour and then removed and kept in the ice box. The relative viscosity of this solution should be about 4. It varies, however, considerably with different casein preparations and especially with the length and temperature of heating. If the solution is not heated the viscosity is much higher but changes quite rapidly at 35°C. which necessitates a large and uncertain correction. The determinations made with the same casein solution may be reproduced within ± 5 per cent but different casein solutions prepared from various samples of casein may give values differing 100 per cent, especially in the value for the positive viscosity change.

Edestin.—25 gm. of crystalline edestin recrystallized from NaCl at 50°C. are stirred into 500 ml. of water at about 40°C. and 35 ml. M/1 hydrochloric acid added and the solution stirred until the edestin dissolves. The flask is then immersed in boiling water for ½ hour and the solution kept in the ice box. The relative viscosity of this solution should be about 2.2. It remains constant indefinitely in the ice box and changes only very slightly at 35.5°C. The change in viscosity of this solution is very regular and comparative results are accurate to ± 2 or 3 per cent when made with the same edestin solution. As in the case of casein, however, the different solutions prepared from different lots of edestin may give quite different results. The viscosity is also sensitive to the length of time and temperature of heating as in the case of casein.

Milk.—A number of attempts were made to prepare a reproducible casein solution for the measurement of the rennet activity. The results, however, were extremely variable as were determinations made with different samples of milk. Solutions may be prepared from dried milk powder ("Klim") which are quite reproducible when prepared from the same original sample and which do not vary excessively when prepared from different samples. 50 gm. of the milk powder is stirred into 500 ml. of water at about 40°C. 50 ml. of M/1, pH 5 acetate buffer added, and the solution kept in the ice box until wanted. Great care must be taken to avoid contamination with the slightest amount of active enzyme as exceedingly minute quantities of pepsin will cause very marked changes in the viscosity of this solution. The specific viscosity of the solution should be about 1.7. It may be kept for several weeks in the ice box or for several hours at 35.5°C. without variation.

Technique of the Determination.—The methods used for measuring the change in viscosity are the same for all the various solutions and one description will serve for all. Ostwald type viscosimeters are used. If reproducible results with different instruments are to be obtained it is necessary that the time of outflow be long enough to give real viscosity measurements; *i.e.*, at least 20 seconds should be required for the passage of 1 ml. of water. It is convenient to carry out four determinations simultaneously and the subsequent calculations are much simpler if the four viscosimeters used all have the same time for the outflow of water. The ordinary type of viscosimeters supplied by apparatus manufacturers contain 2 ml. with a delivery time of about 70 to 80 seconds. They are clamped vertically in a row in a glass-walled thermostat set at 35.5°C. ± 0.05°C. The pipettes are washed and dried while in position by drawing through water and then acetone. Since the capillaries are rather fine in these instruments it is necessary to filter all solutions through hard filter paper as any small particles such as shreds of filter paper will clog the capillary. This is the most troublesome source of variation in the measurements. It is also necessary to use accurate stop-watches as measurements are made to 0.2 of a second and irregularities in the curve have frequently been traced to erratic stop-watches.

5 ml. of the protein solution is pipetted into a 50 ml. test-tube and allowed to remain in the water bath until it has reached the temperature of the bath. 0.2 ml. of the solution to be tested is then added to the 5 ml. protein solution and the time of addition noted. The tube is shaken and contents poured into the wide arm of the viscosimeter. The liquid is drawn up into the bulb with gentle suction so as to avoid air bubbles and the time of the outflow measured with a stop-watch. The time at which the viscosimeter is about one-half empty is also noted and this time is taken as corresponding to that of the viscosity reading. As soon as the liquid has run through the viscosimeter it is drawn up again and another measurement made. In this way a series of times of outflow are determined at intervals of about 5 or 6 minutes. The specific viscosity corresponding to these readings is then calculated by Equation I and these figures are plotted against the elapsed time.

TABLE I

Calculation of Peptic Activity from Viscosity Time Curves

Method	Casein V^- control	Casein V^-	Casein V^+	Rennet	Edestin V^-	Gelatin V^-
ΔV	0.04	0.11	1.20	0.65	0.038	0.024
V_o	3.14	3.14	3.14	0.64	1.326	1.05
Δt min	10	10	10	24	10	10
$[\text{P.U.}]^{\text{Protein } V}$ 5.2 ml. digestion mixture	0.13	0.35	3.8	4.17	0.285	0.23
Mg. pepsin N per 5 ml. protein solution	0	2×10^{-4}	1×10^{-2}	1×10^{-5}	4×10^{-4}	2×10^{-2}
$[\text{P.U.}]^{\text{Prot. } V}_{\text{mg. pepsin N}}$		1100	380	420,000	700	11.5

Pepsin unit = $[\text{P.U.}]^{\text{Protein } V}$ = % change in additional viscosity per min. = $\dfrac{100\,\Delta V}{V_o \Delta T}$

$$[\text{P.U.}]^{\text{Protein } V} = \text{per cent change in viscosity per min.} = \frac{100\Delta V}{V_o\Delta T} = \frac{100(t''_{s0} - t''_{st})}{(t''_{s0} - t''_{\text{H}_2\text{O}})t}$$

t''_{s0} = outflow time for solution at beginning; this is determined by extrapolating to 0 time;

t''_{st} = outflow time for solution at time t.

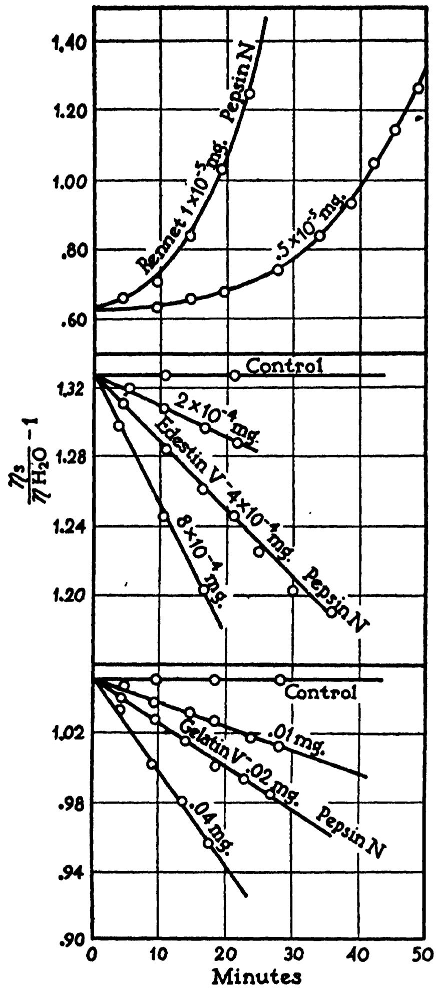


FIG. 1. Change in specific viscosity of milk, edestin, and gelatin solutions with different quantities of crystalline pepsin.

t''_{H_2O} = outflow time for water.

t = time in minutes since enzyme solution is added.

$$\text{Specific viscosity} = V = \frac{t''_s}{t_{H_2O}} - 1$$

An example of the curves obtained in this way with the various proteins are shown

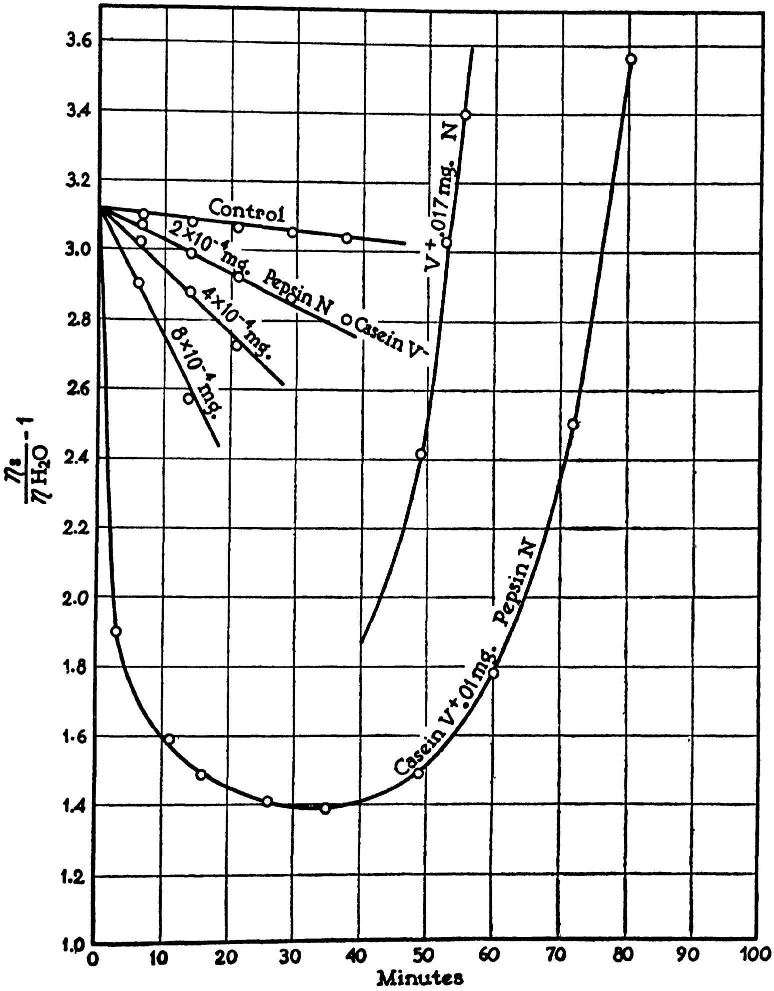


FIG. 2. Changes in viscosity of casein solutions with different quantities of crystalline pepsin.

in Figs. 1 and 2.[1] The number of activity units present are calculated from the slope of these curves as shown in Table I. The calculation has been carried out only for one concentration of enzyme but it can be seen from the slopes of the curves that the rate of change of the viscosity is very nearly proportional to the quality of enzyme present.

---

[1] The curves for the change in viscosity of standard milk previously published (Northrop, J. H., *J. Gen. Physiol.*, 1931, **15**, 41) referred to Parke, Davis U.S.P.

In calculating the value for the increase of viscosity with casein the slope of the viscosity-time curve is measured at the time when the viscosity has returned to its original value. The time required for the viscosity to return to this point could be used as a measure of the speed as has been done by Holter (9), but in this laboratory this figure appears to vary more with different preparations than does the slope of the line.

II. Determination of Activity As Measured by the Production of Non-Protein Nitrogen

Protein Solutions

This determination is made only with casein and edestin and the same solutions are used as for the viscosity measurements.

In order to obtain a figure for the activity which will be independent of the quantity of the enzyme and of the time of the determination it is necessary to choose conditions under which the rate of the reaction is directly proportional to the time and to the quantity of enzyme. This relation holds only in the very first part of the reaction. It is necessary therefore to work either with very low concentrations of enzyme or very short time intervals. Two general methods are possible. The increase in the formation of soluble nitrogen at different time intervals may be determined and the initial rate of increase interpolated from the curve obtained in this way, or the increase in soluble nitrogen at the end of a constant time interval with varying amounts of enzyme may be determined and the rate of formation of soluble nitrogen with small amounts of enzyme interpolated from the initial slope of this curve. The activity for any one preparation of enzyme, as determined by these two methods, agrees within a rather wide experimental error. It is necessary when testing the activity of an unknown solution to measure more than one concentration of enzyme since in order to be significant the specific activity must be independent of the quantity of enzyme used for the determination and this can only be determined by making the test with more than one quantity. This is especially true of the method by which the change after the constant time interval is used since the curves obtained in this way vary considerably with the purity of the enzyme preparation.

1:10,000 pepsin and not to crystalline pepsin as the legend states. The unit of activity for the rennet action (page 42 of the paper just quoted) is also incorrect and should read "per cent change in additional viscosity of standard milk *per minute.*"

Technique of the Determination

I. Determination from the Initial Rate of Digestion.—1.0 ml. samples of the pepsin solution are added to a series of tubes containing 5 ml. of the protein solution at 35.5°C. A blank determination must be made with each pepsin solution by adding 1 ml. of a solution of pepsin which has been boiled for 5 minutes. 5 ml. of 20 per cent trichloracetic acid is then added to the tubes at successive time intervals, the suspensions are filtered, and total nitrogen in 5 ml. of the filter is determined by the micro Kjeldahl method. The quantity of non-protein nitrogen in 6 ml. of the original digestion mixture is calculated from this figure, corrected for the blank determination, and plotted against the time in minutes at which the samples were taken. The activity, by definition, is then determined as the initial slope of these curves and the specific activity of the enzyme sample tested is this slope divided by the quantity of enzyme present. An example of an experiment in which this determination was made with a preparation of crystalline pepsin and with casein and edestin is shown in Fig. 3 and the calculation of the activity is given in Table II. The figure for the specific activity obtained in this way is independent of the quantity of the enzyme used, within the error of the method.

TABLE II

Calculation of Peptic Activity from Non-Protein Nitrogen Time Curves

Protein	Casein			Edestin		
Ml. N/50 N per min. (from initial slope)	0.4	1.1	1.9	0.16	0.34	0.9
Mg. pepsin N	0.022	0.05	0.10	0.010	0.020	0.05
Activity per mg. pepsin N. $[\text{P.U.}]^{\text{Prot. N}}_{\text{mg. N}}$	0.36	0.44	0.38	0.32	0.34	0.36

II. Determination of the Activity from the Quantity of Digestion after 1 Hour.—1.0 ml. of the pepsin solution containing varying amounts of pepsin is added to a series of tubes containing 5.0 ml. of the standard protein solution, and digestion allowed to continue for 1 hour at 35.5°C. The solutions are then analyzed as described for the preceding method and the quantity of soluble nitrogen per 6 ml. of the original digestion mixture plotted against the quantity of pepsin used. The specific activity of the preparation is determined from the initial slope of this curve as in the preceding method. Standard curves, as determined in this way with crystalline pepsin and with casein and edestin, are shown in Fig. 4. The curves for casein and for edestin, with the two particular samples of proteins used in this experiment, are identical. The activity as determined from the initial slope of this curve is about 0.46 milliequivalents per minute per milligram protein nitrogen, which agrees with the value obtained from the time curves[2] by the preceding methods. In other words, 1 mg. of crystalline pepsin nitrogen has an activity of 0.46 units by this method. In determining the activity of an unknown solution the equivalent number of milligrams of crystalline pepsin nitrogen corresponding to the increase in non-protein nitrogen is read from the curve and this figure times 0.46 gives the number of activity units in 1 ml. of the unknown sample.

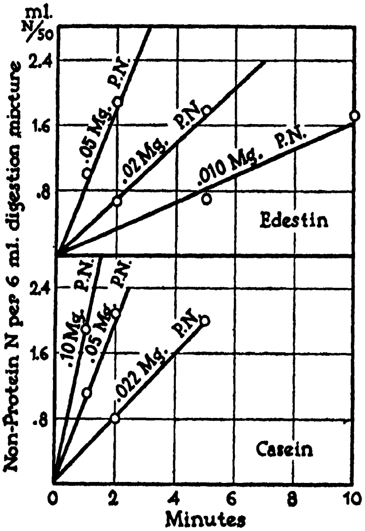


FIG. 3

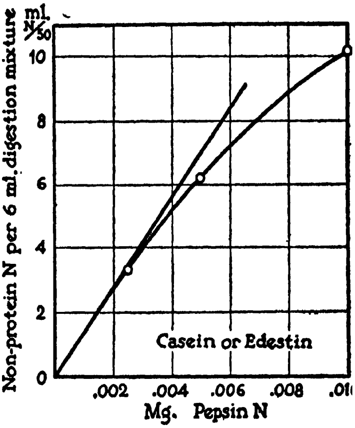


FIG. 4

FIG. 3. Increase in non-protein nitrogen from edestin and casein solutions with different quantities of crystalline pepsin.

FIG. 4. Formation of non-protein nitrogen from casein or edestin after 1 hour at 35.5°C. with different quantities of crystalline pepsin.

---

[2] This experiment is simply a result of the fact that the extent of enzyme reactions in general is determined by the product of the enzyme concentration into the time during which it acts. For instance, if E_1 concentration of enzyme acting for T_1 minutes causes the reaction to proceed A units, then any other pair of values of E and T, such that $ET = E_1T_1$, will cause the same amount of reaction, A. The curves for different quantities of enzyme after the same time, and the curve for the same amount of enzyme at different times could, therefore, be combined into one curve in which the amount of digestion was plotted against the product of the enzyme concentration into the time.

III. Method of Determination of Activity by the Increase in Formol Titration

Protein Solutions

Gelatin.—30 gm. air-dry isoelectric gelatin dissolved in about 400 ml. of water at 40°C., 29 ml. of M/1 hydrochloric acid added, and the solution made up to 500 ml. The pH of this solution should be approximately 2.5.

The same edestin and casein solutions were used as for the other methods.

Technique of the Determinations

1. Method of Determining Activity from Time Curves.—1.0 ml. of the pepsin solution is added to a series of test-tubes each containing 5.0 ml. of the standard protein solution. At various time intervals 5.0 ml. of strong sodium hydroxide is added to the mixture. The strength of the sodium hydroxide is adjusted by trial until 5.0 ml. is just sufficient to bring the solution to a faint pink to phenolphthalein. The alkali should be run carefully down the side of the tube so as not to mix with the protein solution. After the 5 ml. of sodium hydroxide has been added the tube is shaken quickly. This procedure prevents the formation of large clumps of precipitated protein. The tube is then shaken gently until all the protein precipitate is dissolved; 1.0 ml. of 40 per cent formaldehyde solution is then added and the solution titrated to match the standard solution with N/50 sodium hydroxide.

TABLE III

Calculation of Peptic Activity of Crystalline Pepsin by Formation of Non-Protein Nitrogen from Initial Slope of 1 Hour Curves

Protein	Casein	Edestin
Ml. N/50 N per hr. per 6.0 ml. solution (from initial slope)....	7.0	7.0
$\approx$ milliequivalents per min. = $[\text{P.U.}]^{\text{Prot. }S}$..............	0.0023	0.0023
Mg. pepsin N..	0.005	0.005
Activity per mg. pepsin N. $[\text{P.U.}]^{\text{Prot. }S}_{\text{mg. N}}$..................	0.46	0.46

A blank determination is carried out with a sample of the pepsin solution which has been inactivated by boiling.

Preparation of the Titration Standard (10).—The greatest difficulty with the formol titration, as usually carried out, is due to the fact that the color of the solution does not exactly match the standard. This difficulty can be avoided by making the standard out of another sample of the solution itself. To prepare such a standard a sample is run in the same way as for an actual determination. 1 drop of 0.1 per cent phenolphthalein and a large excess of alkali is added so that the indicator develops its maximum color. In titrating the unknown, 1 drop of 0.5 per cent phenolphthalein is used and the titration continued until the unknown exactly matches the standard. By this method the end-point can usually be determined to less than ± 0.05 ml. N/50 sodium hydroxide. The difficulty with this method is that the total titration is very large so that if it were carried on entirely with fiftieth normal alkali a large volume of alkali would be required and the end-point would be very uncertain. This difficulty is avoided by using a small volume of concentrated alkali to neutralize most of the acid and finishing the titration with dilute alkali. It is necessary to measure the concentrated alkali to 0.01 ml. which can best be done by means of a delivery pipette graduated both above and below the bulb. It is also necessary to measure the 5 ml. of protein solution with the same accuracy since a small error in this volume affects the final titration value very markedly owing to the high acidity of the solution. Owing to changes in the viscosity of the protein solution samples cannot be measured with sufficient accuracy if the digestion is carried on in a large volume and 5.0 ml. samples pipetted out at different times.

TABLE IV

Calculation of Peptic Activity from Formol Titration Time Curves

Protein	Gelatin				Casein				Edestin		
Ml. N/50 formol titration per min.	0.05	0.075	0.10	0.17	0.07	0.10	0.18	0.40	0.05	0.10	0.25
Mg. pepsin nitrogen	0.60	1.0	2.0	3.0	0.012	0.020	0.04	0.09	0.005	0.011	0.028
Milliequivalents titration per min. per mg. pepsin N $[\text{P.U.}]^{\text{Prot. }F}_{\text{mg. N}}$	0.0016	0.0015	0.0010	0.0011	0.11	0.10	0.09	0.09	0.20	0.18	0.18

TABLE V

Calculation of Peptic Activity of Crystalline Pepsin by Formol Titration from Initial Slope of 1 Hour Curves

Protein	Casein	Edestin	Gelatin
Ml. N/50 per hr. per 6.0 ml. digestion mixture (from initial slope)	2.2	2.0	0.5
≈ milliequivalents per min. = $[\text{P.U.}]^{\text{Prot. }F}$	0.0007	0.00066	0.00017
Mg. pepsin nitrogen	0.01	0.004	0.2
Activity per mg. pepsin N. $[\text{P.U.}]^{\text{Prot. }F}_{\text{mg. N}}$	0.07	0.16	0.00085

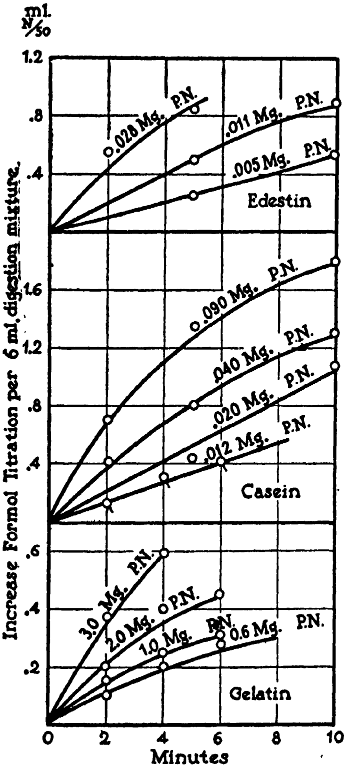


FIG. 5

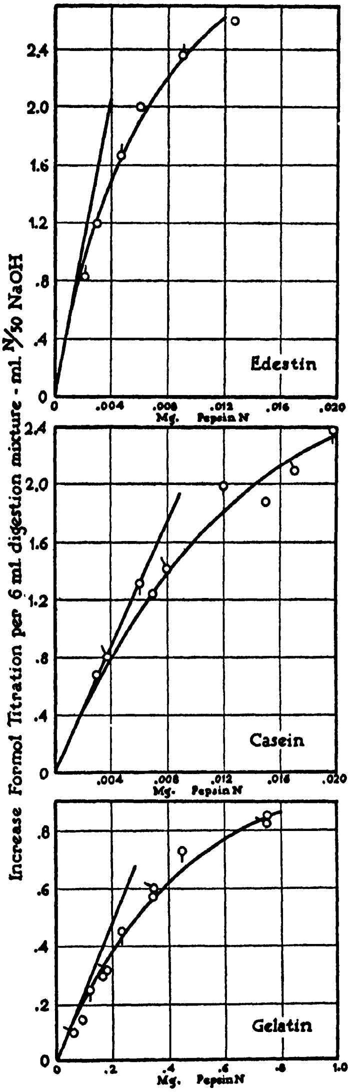


FIG. 6

FIG. 5. Increase in formol titration of gelatin, casein, and edestin with different quantities of crystalline pepsin.

FIG. 6. Increase in the formol titration of gelatin, casein, and edestin after 1 hour with different quantities of crystalline pepsin.

TABLE VI

Summary of Activity of Various Crystalline Pepsin Preparations with Different Protein Solutions

Date	Pepsin	Protein solution	Methods										
			Gel. V^-	Cas. V^-	Ed. V^-	Cas. V^+	Rent.	Cas. *S.*	Ed. *S*	Cas. *F.*	Ed. *F.*	Gel. *F.*	Hb
			$[PU]_{\text{mg. N}}$										
Apr. 18, 1931	C P #44	Apr. 15, 1931	13	1200	570	150	28×10^4	0.44	0.400	0.090	0.08	0.0011	0.18
		Mar. 21, 1932				400	69×10^4			0.14	0.13		
Mar. 22, 1932	# 1	Mar. 20, 1932	15	1250	700	190		0.40	0.34	0.100	0.16		
May 14, 1932	5/9/32	Apr. 22, 1932	12	1100	800	400	42×10^4	0.42	0.42	0.10	0.08	0.0010	0.19
	C P #2	Mar. 20, 1932	12	1200	700	140	21×10^4	0.42	0.42	0.10			
			10	1500		200	29			0.10	0.15		
Mar. 23, 1932	S P #3	Mar. 21, 1932	13	1000	880	400	71×10^4	0.47	0.40	0.16	0.15	0.0020	0.18
			Mg. pepsin protein N per ml. of enzyme solution used										
			0.10	0.001	0.002	0.02–0.05	0.0001	0.005	0.005	0.01	0.01	0.20	0.0025

2. *Method of Determining Activity from the Titration Values.*—The increase in the titration per 6.0 ml. of the digestion mixture is plotted against the time of digestion and the activity determined from the initial slope of this curve exactly as in the case of the non-protein nitrogen method. A series of determinations made in this way with crystalline pepsin and with casein, gelatin, and edestin are shown in Fig. 5 and Table IV. The specific activity is independent of the quantity of enzyme within the rather wide error of the method.

3. *Method of Determining the Activity from the Amount of Digestion after 1 Hour at 35.5°C.*—A standard curve for the increase in the formol titration after 1 hour with various quantities of the enzyme is prepared in the same way as for the non-protein nitrogen method. The specific activity is calculated from the initial slope, as described above, and determinations with an unknown sample are made by interpolating from the curve as described for the previous method. The curves obtained with crystalline pepsin and with casein, edestin, and gelatin are shown in Fig. 6 and the calculation of the specific activity in Table V.

Accuracy of the Formol and Non-Protein Nitrogen Methods

In comparative experiments with the same protein preparations the activity of an unknown solution may be determined with an accuracy of about ± 5 per cent for one determination but different casein and especially different edestin preparations vary, for some unexplained reason, quite considerably. The value obtained with gelatin is much more constant with different gelatin preparations but the experimental error is greater than with casein or edestin owing to the small titration value. A summary of the specific activity of several preparations of crystalline pepsin as determined by these methods is shown in Table VI. The last line of the table gives the concentration of the solution of crystalline pepsin used for the determination. The table shows that the activity of the various preparations as measured by any one method is quite constant except for the casein V^+ method and the casein and edestin formol titration. With these methods the values for the activity found with the last preparation is considerably higher than that found for the first preparation reported. A sample of the first preparation had been preserved in glycerin in the ice box and when measured with the protein solutions used to determine the activity of the last preparation, showed the same activity. The variation, therefore, is due to variation in the protein solutions rather than in the pepsin. The activity units used in this table are the same as those used in the paper on the preparation of gelatinase (3) except that the present ones are expressed as activity per milligram pepsin nitrogen while in the paper referred to the activity was expressed as per gram nitrogen. They differ, however, from the activity units used in the first paper on the activity of crystalline pepsin (11) since in that case the activity, as determined by changes in viscosity, was arbitrarily assumed to be numerically equal to that determined by formol titration.

SUMMARY

Experimental methods are described for determining the activity of pepsin preparations by means of changes in the viscosity of gelatin, casein, edestin, and powdered milk solutions, and by the rate of formation of non-protein nitrogen from casein and edestin solutions, or by the increase in formol titration of casein, edestin or gelatin.

Activity units for pepsin are defined in terms of these measurements.

BIBLIOGRAPHY

1. Euler, V., Chemie der Enzyme, München, Bergman, 3rd edition, 1st part, 1925, 17.
2. Willstätter, R., Waldschmitz-Leitz, E., Dunaiturria, S., and Künstner, G., *Z. physiol. Chem.*, 1926, **161**, 191.
3. Northrop, J. H., *J. Gen. Physiol.*, 1931, **15**, 29.
4. Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1923, **5**, 353.
5. Northrop, J. H., *J. Gen. Physiol.*, 1929, **12**, 529.
6. Rona, P., and Oelkers, H. A., *Biochem. Z.*, 1930, **217**, 50.
7. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1932, **16**, 59.
8. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1928, **11**, 477.
9. Holter, H., *Z. physiol. Chem.*, 1931, **196**, 1.
10. Northrop, J. H., *J. Gen. Physiol.*, 1926, **9**, 767.
11. Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739; *Ergebn. Enzymforsch.*, 1932, **1**, 302.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1932,
Vol. 16, No. 1, pp. 59–63]

THE ESTIMATION OF PEPSIN WITH HEMOGLOBIN

BY M. L. ANSON AND A. E. MIRSKY

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Hospital of The Rockefeller Institute for Medical Research, New York)

(Accepted for publication, May 20, 1932)

A number of methods have been described for the estimation of peptic activity, many of which are accurate and convenient for comparative experiments but none of which give results which are accurately reproducible with different preparations of the protein used. This variation is due to the difficulty involved in obtaining reproducible protein preparations and keeping them unchanged.

Hemoglobin has been chosen as the protein substrate for the estimation of pepsin because it is easily prepared in large quantities, because it can be stored in solution for a long time without change, because it is rapidly digested, and because the rate at which it is digested by a given pepsin solution does not vary from one hemoglobin preparation to another. Not only is hemoglobin a reproducible protein which can be brought to reproducible conditions but the conditions of digestion have been so chosen that reasonable variations in these conditions have little effect on the amount of digestion.

In general the procedure used to estimate pepsin is this. A pepsin solution is added to acidified hemoglobin. After 5 minutes trichloracetic acid is added. The resulting precipitate which contains all the pigment and all the undigested hemoglobin is filtered off. The filtrate contains an amount of digested hemoglobin which is a measure of the amount of pepsin used. This digested hemoglobin is estimated by the blue color it gives with the phenol reagent, which reacts with tyrosine, tryptophane and cysteine groups, tyrosine being used as a standard. The intensity of this color is proportional to the amount of enzyme used and to the time of digestion.

Definition of Activity $[\text{P. U.}]^{\text{Hb}}$.—One unit of activity is defined as the formation per minute at 35.5°C. in 6 ml. of the standard digestion mixture of an amount of digested hemoglobin not precipitable by trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of digested hemoglobin when contained in 6.0 ml. of the standard digestion mixture (see Northrop, 1932, for a discussion of the definition of pepsin units).

In detail the procedure is this: 5 ml. of a 2 per cent solution of dialyzed ox carbon monoxide hemoglobin in 0.06 N HCl are pipetted into a 175 × 20 mm. test tube and brought to 35.5°C. or 25°C. 1 ml. of enzyme solution is added with an Ostwald pipette, which procedure takes about 4 seconds. The middle of the pipetting period is taken as the time of beginning. To mix the hemoglobin and enzyme solution the test-tube is whirled. After 5 minutes, 10 ml. of 4 per cent trichloracetic acid are poured into the hemoglobin solution from another test-tube, the suspension is poured back and forth from one test-tube to another, and filtered through fine paper. To 3 ml. of the filtrate in a 50 ml. Erlenmeyer flask are added 20 ml. of water, 1 ml. of 3.85 N NaOH, 1 ml. of the phenol reagent (Folin and Ciocalteau, 1927). Some of this NaOH is to neutralize trichloracetic acid. The phenol reagent and NaOH are added from automatic pipettes (A. H. Thomas Co.—No. 8210). The standard is made up from 3 ml. 0.1 N HCl containing 0.15 mg. tyrosine.[1] After 5 to 10 minutes, the blue colors are compared, the standard solution being set at 20, and the reading for the unknown solution being called X.

In addition to 0.15 mg. tyrosine, the standard solution contains from the reagents an amount of color-producing substance equivalent to 0.015 mg. tyrosine.[2] The color-producing substance in the 3 ml. of filtrate is therefore equivalent to $\frac{20}{X}(0.15 + 0.015)$ mg. tyrosine. Of this 0.015 mg. are due to the color-producing substance in the reagents and 0.01 mg. to the color-producing substance present in the trichloracetic acid filtrate even when no enzyme is added. The digested hemoglobin in the 3 ml. of filtrate is therefore equivalent to $\frac{20}{X}$ (0.15 + 0.015) −0.015 −0.01 mg. tyrosine. This value must be multiplied by $\frac{16}{3}$ to obtain the digested hemoglobin in the whole 16 ml. of filtrate instead of in the 3 ml. taken for analysis; it must be divided by 5 to obtain the amount of non-precipitable digested hemoglobin produced in 1 minute instead of in 5 minutes; and it must be divided by 181, the molecular weight of tyrosine, to obtain the tyrosine equivalent as milliequivalents instead of as milligrams. The relation between the number of pepsin units in 1 ml. of enzyme solution and the colorimeter reading *X* is thus:

---

[1] Copper sulfate can be used as a standard instead of the blue solution obtained from tyrosine with the phenol reagent. The colors match if a red color filter is used.

[2] The reagent blank is not measurable. It is deduced from the fact that colors given by tyrosine solutions in the relatively low range of concentrations used are not proportional to the tyrosine concentrations—doubling the tyrosine concentration decreases by some 5 per cent the color given per unit amount of tyrosine. Whether this deduction of a reagent blank equivalent to 0.015 mg. tyrosine is correct or not makes no significant difference to the validity of the calculations which merely provide a correction for the small deviation from proportionality.

$$\text{P. U.} = \left[\frac{20}{X}(0.15 + 0.015) - 0.015 - 0.01\right]\frac{16}{3} \times \frac{1}{5} \times \frac{1}{181} = \frac{0.0195}{X} - 0.000147$$

If the digestion is carried out at 25°C. at which temperature it is 1.82 times slower than at 35.5°C. the right hand expression must be multiplied by 1.82 since the unit of activity is defined as the rate of reaction at 35.5°C. By the use of a curve in which P. U. is plotted against *X*, one can in practice avoid all calculations.

In the table are recorded actual estimations in terms of pepsin units of the activities of a series of solutions of five times recrystallized pepsin. The constancy of the specific activity, that is the activity per mg. protein nitrogen per ml., is an expression of the fact that the amount of non-precipitable color-forming substance liberated from hemoglobin is proportional to the pepsin concentration.

Protein N per ml. pepsin solution	Activity units $[\text{P. U.}]^{\text{Hb}}$		Specific activity $\left[\text{P. U.}\right]^{\text{Hb}}_{\text{mg. N}}$	
	35.5°C	25°C.	35.5°C.	25°C.
mg.				
0.0180		0.00326		0.181
0.0144		0.00260		0.180
0.0108	0.00194	0.00206	0.180	0.191
0.0072	0.00134	0.00136	0.185	0.189
0.0054	0.00100	0.00103	0.185	0.191
0.0036	0.00065	0.00064	0.180	0.178
0.0018	0.00034		0.189	
			Average...........0.184	

The Preparation of Hemoglobin.—Carbon monoxide is bubbled through whipped ox blood. The blood is centrifuged and the serum and white cells siphoned off. After the corpuscles are washed four times with cold 0.9 per cent sodium chloride, an equal volume of water and a sixth the total volume of toluol are added (Heidelberger, 1922). Carbon monoxide is bubbled through the solution which is then vigorously shaken and allowed to stand in the cold overnight. Next morning the hemoglobin layer is siphoned off, mixed gently with a tenth the volume of centrifuged alumina cream (Tracy and Welker, 1915), centrifuged, and then filtered through coarse paper. Finally the hemoglobin solution is dialyzed overnight in the cold in a shaking dialyzer (Kunitz and Simms, 1928) and stored in the cold under carbon monoxide with toluol as a preservative.

The concentration of hemoglobin is estimated by the Kjeldahl method, the nitrogen content being taken as 17.7 per cent. Hemoglobin is digested with greater difficulty than are the other common proteins. When copper is used as a catalyst, digestion is not complete. When mercury is used, digestion is complete but a preliminary evaporation is necessary to avoid serious foaming. With selenium oxychloride (Lauro, 1931) digestion is rapid and complete and no preliminary evaporation is needed. The concentration of the thrice recrystallized tyrosine is likewise determined by the Kjeldahl method—nitrogen content, 7.74 per cent.

To make up the acid solution of hemoglobin three volumes of 0.1 N HCl are added to two volumes of 5 per cent hemoglobin. This acidity (0.06 N) is sufficient to give the maximum rate of digestion and is safely on the acid side of the region in which the rate of digestion is sensitive to small changes in the hydrogen ion concentration. Changing the HCl concentration to 0.035 N or to 0.08 N does not change the rate of digestion more than 5 per cent. Hemoglobin is hydrolyzed very slowly in 0.06 N HCl. Acid solutions which have stood in the cold for 10 days have been used for the estimation of pepsin with results the same as those obtained from freshly acidified hemoglobin.

Increasing the hemoglobin concentration from 2 per cent to 3 per cent or decreasing it to 1.5 per cent has no significant effect on the rate of digestion.

Trichloracetic acid in the concentration chosen (2.5 per cent) gives a precipitate which filters rapidly. If the trichloracetic acid concentration is decreased 20 per cent, some 10 per cent more color-producing material is left unprecipitated. If the trichloracetic acid concentration is increased 20 per cent some few per cent less color-producing material is left unprecipitated. 10 per cent trichloracetic acid, as well as other so called protein precipitants, precipitates a great deal of the digested hemoglobin not precipitated by 2.5 per cent trichloracetic acid; so that the digested hemoglobin could probably be estimated nephelometrically as well as colorimetrically.

In general, then, the reproducibility of the amount of digestion obtained with a given pepsin solution from different hemoglobin preparations depends essentially on the reproducibility of hemoglobin as a protein rather than on accuracy in fixing the concentrations of hemoglobin, hydrochloric acid, and trichloracetic acid, although in practice these concentrations are fixed accurately with little trouble.

The relations between the hemoglobin pepsin unit and other pepsin units are given by Northrop (1932).

A method will be described later for the estimation with hemoglobin of active, native trypsin in the presence of inactive, denatured trypsin, together with a discussion of the peculiar problems involved in the estimation of trypsin, in particular the prevention of the reversal of inactivation and denaturation. By measurements of tryptic digestion, furthermore, one can estimate denatured hemoglobin in the presence of native hemoglobin, for native hemoglobin is not digested by trypsin while denatured hemoglobin is digested at a rate which depends on its concentration.

REFERENCES

Folin, O., and Ciocalteau, V., 1927, *J. Biol. Chem.*, **73**, 627.
Heidelberger, M., 1922, *J. Biol. Chem.*, **53**, 31.
Kunitz, M., and Simms, H., 1928, *J. Gen. Physiol.*, **11**, 641.
Lauro, M., 1931, *Ind. and Eng. Chem.*, *Analytical Edition*, **3**, 401.
Northrop, J. H., 1932, *J. Gen. Physiol.*, **16**, 41.
Tracy, G., and Welker, W., 1915, *J. Biol. Chem.*, **22**, 55.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1932,
Vol. 16, No. 1, pp. 147–156]

PROTOPLASMIC POTENTIALS IN HALICYSTIS

II. THE EFFECTS OF POTASSIUM ON TWO SPECIES WITH DIFFERENT SAPS

BY L. R. BLINKS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, May 20, 1932)

In studying the potential differences in plant cells it is desirable to alter the solutions in contact with the protoplasm, not only at the outer surface, but at the inner or vacuolar surface as well, since this may differ from the outer in its properties. Such interior alteration may be done experimentally (to be described in other papers of this series), or by the comparison of different species essentially alike except for different vacuolar saps. A striking case of such a natural experiment is available in the two known American species of *Halicystis*. *H. Osterhoutii* of Bermuda has a cell sap differing only slightly from sea water in composition;[1] *H. ovalis*, of the Pacific Coast, on the contrary accumulates potassium (as chloride) to some thirty times its concentration in the sea water.[2] These two species differ otherwise only in minor details of morphology.[3,4] They thus offer an excellent opportunity to test the internal effect of a salt so generally active bioelectrically as potassium chloride.

Has *H. ovalis* a relatively high positive potential, like *Nitella*, as a result of the high potassium content of its sap? Or does it, like another marine plant, *Valonia*, have a small negative potential? What part of the observed potential is due to the potassium concentration gradient, what to an independent, internal gradient or asymmetry in the protoplasm? Finally, of critical importance to the theory of accumulation of electrolytes, does potassium ion have a higher mobility in the protoplasm of *H. ovalis* where potassium is accumulated, than in *H. Osterhoutii* where it is not accumulated?

---

[1] Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929–30, **13**, 733.

[2] Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 209.

[3] Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, 1930–31, **57**, 389.

[4] Smith, G. M., Observations on some siphonaceous green algae of the Monterey Peninsula, in Contributions to marine biology, Stanford University Press, 1930, 222.

In answer to these questions, comparisons are given in this paper between the potentials observed with *H. Osterhoutii* and *H. ovalis* under similar experimental conditions.

Method

The method of impalement and measurement has been described elsewhere in more detail.[5] Fine glass capillaries, filled with artificial sap, were inserted from above into the cells, which were supported below on glass rings.[6] Measurement was by compensation, the null instrument being a vacuum tube electrometer drawing no appreciable current. Saturated KCl-calomel electrodes with KCl salt bridges were employed for accurate measurements. For convenience in rapidly measuring a series of impaled cells, silver wire electrodes were sometimes used. These were frequently recoated electrolytically with chloride. They gave potentials not over 1 mv. apart when both were in equilibrium with sea water, or 2

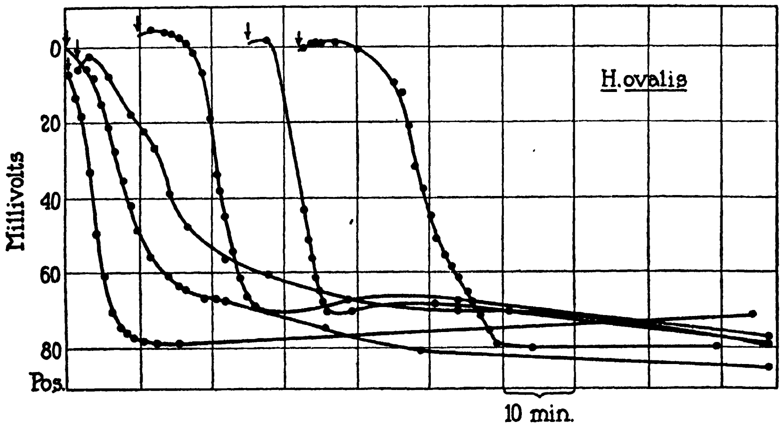


FIG. 1. Potential difference across the protoplasm of *Halicystis ovalis* immediately after impalement. The growth of P.D. in *H. Osterhoutii* is essentially the same. Cells in sea water.

---

[5] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 223.

[6] Damon, E. B., *J. Gen. Physiol.*, 1931–32, **15**, 525, gives an illustration of this arrangement for supporting cells.

or 3 mv. apart when one was in artificial sap. These corrections were applied to the readings. When natural sap was applied externally to the cells, it was found that the silver electrode could not be immersed directly therein without giving very erratic readings; the chloride was possibly reduced by some organic substance of the sap. (This would constitute a very serious objection to the insertion of silver electrodes, such as Sen's microelectrode,[7] directly into cells.) A guard tube was therefore employed in these cases, the electrode being kept in artificial sap, which communicated with the natural sap through an agar-filled capillary.

The cells were usually between 5 and 10 mm. in diameter, and frequently had a bit of the *Lithothamnium* substrate still attached to the pedicel. Cells in active reproduction were not used, but the potentials of those impaled a day or two after reproduction did not differ markedly from those which had recovered for longer periods. Subsequent reproduction did not usually occur while the cells were impaled, although they might live as long as 2 or 3 weeks, *i.e.*, over one reproductive period.

Since neither calcium nor magnesium content was included in the analyses[2,8] of *H. ovalis* sap available at the time of experimentation, artificial sap could only be made up approximately. This was done by mixing 1.5 parts of 0.5 M KCl with 1 part of sea water, giving the ratio K ÷ Na = 1.5, found by Brooks.[2] That this was a fair approximation was shown by the similar behavior of such artificial sap and of natural sap extracted from the cells.

Throughout this paper the potentials given are those of the outside solution, this being positive to the sap in the external circuit (positive current tending to flow outward across the protoplasm). In the figures these positive potentials are plotted below the zero line in conformity with the convention for *Valonia* and *Nitella* in this Laboratory. This is the reverse of the plotting used in the first paper[5] of this series. Change of solution is indicated by arrows.

Comparison of Potentials in Sea Water

Immediately after the impalement of cells of either species of *Halicystis*, there is zero potential difference, or at most a few millivolts, measurable between sap and sea water. But (as shown for *H. ovalis* in Fig. 1), this rapidly increases, reaching in the course of 30 to 60 minutes a maximum which is then maintained during the life of the cell,—as long as 2 or 3 weeks. There are subsequent small fluctuations from day to day, but the potential of healthy cells seldom goes below 50 mv. (except when conditions are experimentally altered), nor does it much exceed 90 mv. In general the potential of *H. Osterhoutii* lies between 60 and 75 mv.; that of *H. ovalis* somewhat higher, between 70 and 85 mv. The figures given in Table I were obtained from a group of several hundred readings for each species (all after at least 1 day's impalement); partly from routine determinations of all cells impaled at any given time (taken each day or two), partly from the initial readings obtained with cells before experimentation. The readings below 50 mv. were discarded in computing the mean, since almost invariably these were accompanied with evident injury, the cell usually dying the same day, with rapid decline of potential to zero. They thus represent, in the author's opinion, as nearly as possible a fair sample of healthy cells of each species. The

---

[7] Sen, B., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 310.

[8] Hollenberg, G. J., *J. Gen. Physiol.*, 1931–32, **15**, 751.

TABLE I

Comparison of Potential Differences across the Protoplasm of Impaled Cells of Two Species of Halicystis

	Mean value	Average deviation of readings	No. of readings	Probable error of mean
	mv.	*mv.*		*mv.*
H. ovalis (1931)	79.7	±5.0	270	±0.25
H. Osterhoutii (1930)	65.6	±9.1	150	±0.63
(1931–32)	68.4	±4.2	130	±0.3

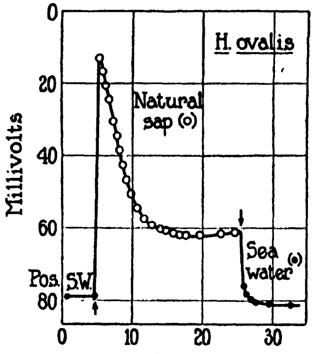


FIG. 2

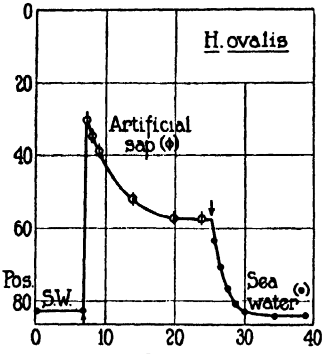


FIG. 3

FIG. 2. Potential difference across the protoplasm of *H. ovalis* in sea water and in freshly extracted natural sap.

FIG. 3. Potential difference across the protoplasm of *H. ovalis* in sea water and in artificial sap.

values for *H. Osterhoutii* for 1931–32 should be given more weight than those for 1930, since the technique was better, and the average deviation less. We assume therefore that the mean potential difference across the protoplasm of *H. ovalis* is about 12 mv. more positive than across that of *H. Osterhoutii*, each being immersed in the sea water of its accustomed environment and measured under the same general conditions of experimentation, *e.g.* temperature (15–20°C.), illumination (diffuse north light in the laboratory), pH (8.1), etc.

The External Application of Potassium Chloride

Since the higher positive potential occurs in *H. ovalis*, the species containing the higher concentration of KCl, it is interesting to know how much of it is due to that salt. This is determined by abolishing the gradient of KCl concentration across the protoplasm. Solutions containing 0.3 M KCl (approximately the amount in the sap of *H. ovalis*) were applied (maintaining the necessary physiological balance with salts of calcium and magnesium). The effects of applying natural sap and artificial sap to *H. ovalis* are shown in Figs. 2 to 5. There is first a rapid decrease of potential, followed by a slower increase during which the potential is again built up, usually to about 60 mv., but in a few cases nearly equaling the original potential in sea water. Since this occurs when the potassium concentration is equal on both

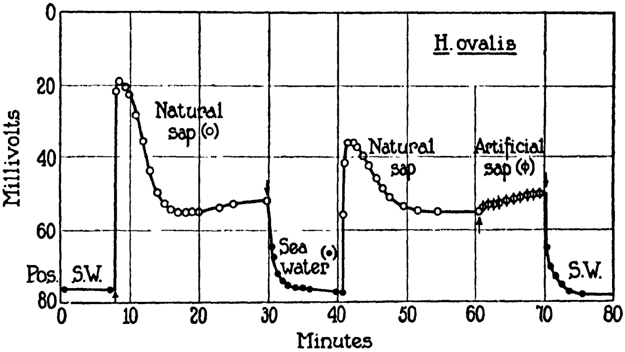


FIG. 4. Potential difference across the protoplasm of *H. ovalis* in sea water and during successive applications of natural sap. Artificial sap is substituted for natural sap during the second exposure.

sides of the protoplasm, the observed potential in sea water must be largely protoplasmic, the potassium potential contributing at equilibrium only 20 mv. out of nearly 80, and often less. Very similar

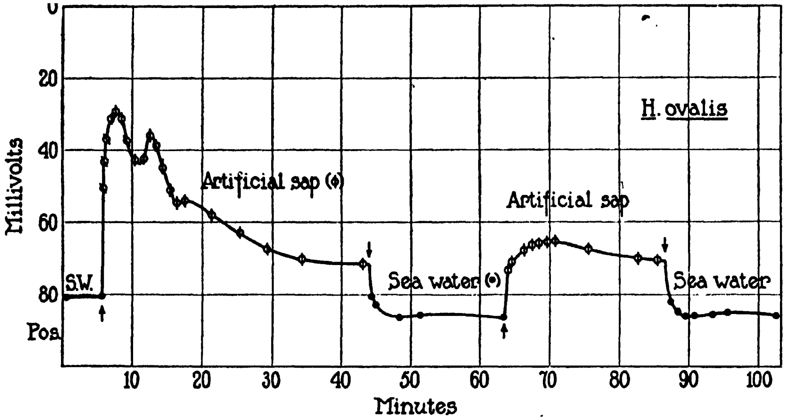


FIG. 5. Potential difference across the protoplasm of *H. ovalis* in sea water and artificial sap, showing reduced effect of second exposure.

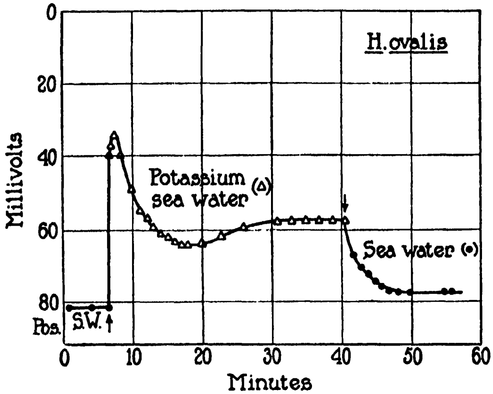


FIG. 6. Potential difference across the protoplasm of *H. ovalis* in normal sea water and in potassium sea water (artificial sea water in which potassium is substituted for sodium).

curves (Fig. 6) are obtained when potassium sea water (artificial sea water in which the sodium is entirely replaced by potassium so that KCl = 0.5 M) is applied externally, a large positive potential persisting even though the gradient of potassium concentration is now reversed.

However, because the gradient of potassium chloride contributes only partially to the observed steady potential across the protoplasm, it is not to be assumed that K$^+$ ion has a low mobility therein (or one nearly equal to that of the anion). On the contrary, if KCl diffused extremely readily into the protoplasm, so that the latter soon contained nearly as high a concentration of this salt as the sap, the potential across the inner surface at least would be greatly reduced

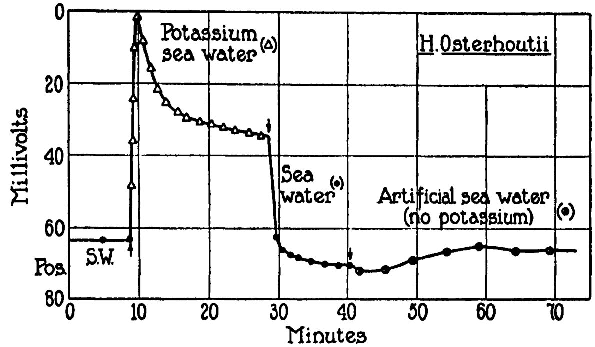


FIG. 7. Potential difference across the protoplasm of *H. Osterhoutii* in normal sea water and in artificial sea water containing (*a*) no sodium, (*b*) no potassium.

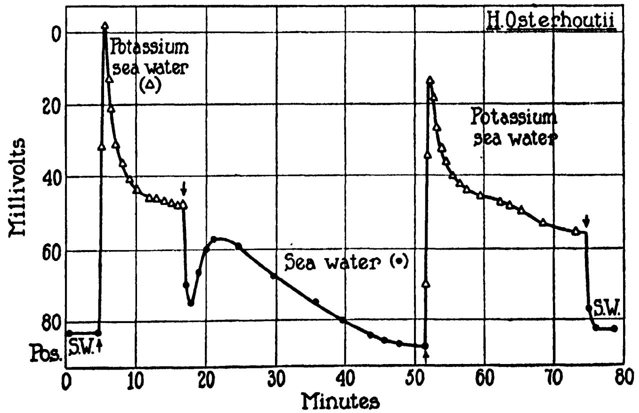


FIG. 8. Potential difference across the protoplasm of *H. Osterhoutii* in normal sea water and in potassium sea water (sodium replaced by potassium).

(*cf.* Damon[9]). That this may happen is indicated by the behavior when potassium chloride is applied externally, the rapid lowering of potential probably being due to the diffusion potential, the recovery to the disappearance of the gradient as diffusion proceeds. From the maximum height of the upward cusp, therefore, could be calculated at least a minimum mobility for potassium ion, relative to chloride, and this would have rather a large value, just as in *Valonia.*[9] But except where the curve flattens out well, the author is inclined to believe such calculations rather doubtful in significance, since the height may depend largely upon the speed with which one solution is substituted for another.

A decreased effect of solutions containing potassium chloride is seen when one exposure quickly follows another, with only a short intervening sojourn in sea water (Figs. 4 and 5). This might also indicate that potassium has entered the protoplasm to a considerable extent during the first exposure, and has not rapidly diffused out again.

In order to compare the two species, the same experiments were performed with *H. Osterhoutii,* in which potassium has essentially the same effect. Figs. 7 and 8 show such experiments. There is again a large but temporary decrease of the positive potential, followed by a rise in which the potential is built up to a value about 20 or 30 mv. lower than the original value. Here again we may assume that the cusp represents the diffusion potential of KCl, and the recovery, the diffusion of KCl into the protoplasm (and probably also into the vacuole). The behavior is practically identical in the two species except that potassium ion may have even a higher mobility in *H. Osterhoutii.*

The Interior Alteration of Potassium Content

We might therefore assume that if the sap of *H. Osterhoutii* could in some manner be increased in potassium content, the potential across the protoplasm would become higher. This assumption has been recently justified by a simple method of vacuolar irrigation, which will be more fully described in another paper dealing with the experimental alteration of cell sap. For present purposes it may be said that it has proved possible to mix the fresh natural sap of *H.*

---

[9] Damon, E. B., *J. Gen. Physiol.*, 1929–30, **13**, 207, and unpublished results.

Osterhoutii with an equal volume of 0.6 M KCl solution, thereby raising its potassium content approximately to that of *H. ovalis.* When this mixture is irrigated through the cells of *H. Osterhoutii,* the potential across the protoplasm often rises from 65 mv. to 80 mv. or more, and in some cases has remained at 75 mv. for several days thereafter. This indicates that potassium has much the same effect on the inner surfaces of the protoplasm of both species, and that the higher potential of *H. ovalis* is probably largely due to the higher potassium content of its sap. The reverse experiment, remaining to prove this, would involve the lowering of KCl in the latter species. But to bring this down to the level of *H. Osterhoutii* would require a 30-fold dilution of natural sap with isotonic NaCl, etc., an amount which the cells have so far not been found to endure. Were both species simultaneously available, the fresh sap of *H. Osterhoutii* could probably be substituted in the cells of *H. ovalis* without difficulty, just as that of *Valonia* can be irrigated through *H. Osterhoutii.* It is hoped that this experiment may be performed in the future.

CONCLUSION

It should be pointed out that these experiments do not favor the hypothesis that potassium is accumulated more in some plants than in others because its ionic mobility is higher in their protoplasm. In the two species of *Halicystis,* the electrical effects of potassium (hence probably its ionic mobility) are almost identical, or slightly larger in *H. Osterhoutii.* Yet *H. ovalis* accumulates KCl some 30-fold, *H. Osterhoutii* not at all.

For their kind invitation to work at Pacific Grove, and for the use of the facilities of the Jacques Loeb Laboratory, the author is deeply indebted to Dr. W. K. Fisher, Director of the Hopkins Marine Station, and to Dr. G. J. Peirce, executive head of the Department of Botany, Stanford University.

SUMMARY

The potential difference across the protoplasm of impaled cells of two American species of *Halicystis* is compared. The mean value for *H. Osterhoutii* is 68.4 mv.; that for *H. ovalis* is 79.7 mv., the sea water being positive to the sap in both.

The higher potential of *H. ovalis* is apparently due to the higher concentration of KCl (0.3 M) in its vacuolar sap. When the KCl content of *H. Osterhoutii* sap (normally 0.01 M or less) is experimentally raised to 0.3 M, the potential rises to values about equal to those in *H. ovalis.*

The external application of solutions high in potassium temporarily lowers the potential of both, probably by the high mobility of K^+ ions. But a large potential is soon regained, representing the characteristic potential of the protoplasm. This is about 20 mv. lower than in sea water.

The accumulation of KCl in the sap of *H. ovalis* is apparently not due to the higher mobility of K^+ ion in its protoplasm, since the electrical effects of potassium are practically identical in *H. Osterhoutii,* where KCl is not accumulated.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1932,
Vol. 16, No. 1, pp. 157-163]

THE KINETICS OF PENETRATION

IV. DIFFUSION AGAINST A GROWING POTENTIAL GRADIENT IN MODELS

BY W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Accepted for publication, May 18, 1932)

Cells of *Nitella* in tap water show an outwardly directed E.M.F. of from 50 to 300 millivolts. Previous experiments[1] suggest that this is largely due to potassium ions whose entrance into the vacuole produces an outwardly directed E.M.F. This tends to oppose their further penetration but they nevertheless continue to enter.

Something of this sort seems to happen in certain models recently described.[2] An example is seen in an experiment in which 0.05 M KOH is shaken with a small excess of a mixture of 70 per cent guaiacol and 30 per cent *p*-cresol (which may be called G.C. mixture) which converts most of the KOH to organic salts of potassium. For convenience these organic potassium salts may all be regarded as K-guaiacolate and called K*G*.[3]

The resulting solution is allowed to flow in a steady stream over a layer of G.C. mixture. We may then say that the K*G* passes from the outer solution (*A*) through the non-aqueous layer of G.C. mixture (*B*) and penetrates into the inner solution (*C*), which at the start consisted of distilled water in which CO_2 was bubbling. The latter may be regarded as analogous to the sap of *Nitella* and designated as "artificial sap." *B* will then correspond to the non-aqueous protoplasmic layer and *A* to the external solution bathing the *Nitella* cell.

---

[1] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715; Electrical phenomena in the living cell, in Harvey Lectures, 1929-30, Baltimore, The Williams and Wilkins Co., 1931; *Biol. Rev.*, 1931, **6**, 369.

[2] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, **15**, 667.

[3] This involves no serious error as the K-*p*-cresolate seems to behave like the K-guaiacolate.

On reaching C, KG reacts to form $KHCO_3$ which may accumulate until its concentration reaches 0.63 M. Hence the osmotic pressure in C becomes much greater than in A. The energy required to produce this result comes from the reaction of KG with CO_2. In part this energy finds expression in the form of E.M.F's.

In order to measure the E.M.F.'s produced in the model the apparatus shown in Fig. 1 was employed. With this arrangement we may suppose that a thin layer of II, which we may call l_0, (perhaps only a few molecules thick) will come at once into equilibrium with III and will in consequence have a different composition from the rest of II (which may be called l).

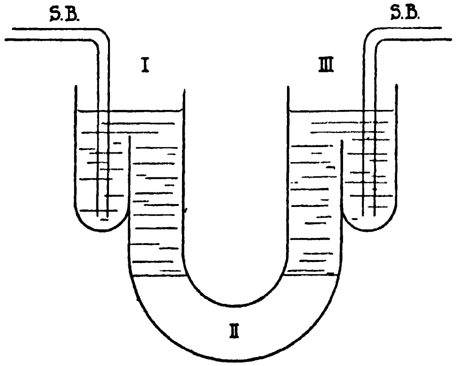
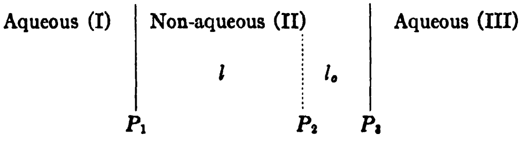


Fig. 1. Aqueous solutions are placed in I and III and a non-aqueous liquid in II. The salt bridges, S.B. (consisting of glass tubes filled with agar made up in saturated KCl solution) were placed at the sides in order that KCl sinking downward might not contaminate the interfaces.

Presumably we have the three potentials shown below, namely,



the phase boundary potentials P_1 and P_3, and the diffusion potential P_2 in the non-aqueous layer.

Unless otherwise stated the procedure was as follows. The aqueous solution in I (Fig. 1) and the non-aqueous solution in II were shaken together and left in contact for 24 hours or more before they were placed in the apparatus. The aqueous solution in III was shaken with a small excess of the non-aqueous solution in II, placed in a separatory funnel and freed from the non-aqueous portion which was discarded. Hence when the solutions were placed in the apparatus there was presumably no exchange of ions between I and II, but ions moved from III into II and set up a diffusion potential[4] in II. If there be any diffusion potential in III due to exit of ions from II it must be small.

To imitate electrical conditions in the model near the start[5] of the experiment aqueous solution from *A* (consisting of 0.05 M K*G*) is placed in I. Non-aqueous solution from *B* (consisting of G.C. mixture containing K*G*) is placed in II. In III we might place distilled water, but as this gives irregular results we prefer to use 0.001 M $KHCO_3$ (which would represent the condition of *C* in Model III[2] a short time after starting the experiment).

This gives a potential of 45 millivolts,[6] I being negative to III in the external circuit (hence *A* is negative to *C* in the model). On bubbling CO_2 in III (without disturbing the interface unduly) K*G* in II begins to combine with CO_2 at the interface to form $KHCO_3$ (which is much more soluble in III than in II). Hence the concentration of $KHCO_3$ in III increases and that of H^+ decreases.

This is illustrated by an experiment in which solution from *A* in a model which had been running for some time was placed in I (this consisted[2] of 0.03 M K*G* + 0.02 M $KHCO_3$). Solution from *B* was placed in II and 0.0001 M $KHCO_3$ in III. At the start the P.D. was 33 millivolts (I being negative to III in the external circuit): this gradually rose to 35 millivolts. Then CO_2 was bubbled in III and in the course of 70 minutes (shown in Fig. 2) III became negative to I in the external circuit so that positive current tended to flow from III through II into I. Stirring[7] II facilitated this change by bringing more K*G* to the interface between II and III. In an experiment in which II was stirred occasionally, III became 24 millivolts negative to I (in the external circuit) in the course of 55 minutes. (When III is stirred for a moment, it becomes temporarily less negative to I because stirring carries $KHCO_3$ away from the interface between II and III.)

---

[4] As a rule the diffusion potentials appear to be much greater in G. C. mixture than in water.

[5] *I.e.*, before much $KHCO_3$ entered *A*.

[6] Average of 43, 48, 35, and 54.

[7] This was done in a U-tube having an upright tube in the center through which a stirrer was inserted in II.

These experiments show that in the model the positive current at the start tends to flow from *A* through *B* into *C* but that later this is reversed so that *C* becomes negative to *A* in the external circuit. This negativity continues to increase until the steady state is reached. To ascertain the negativity in the steady state solutions taken from the model in the steady state were placed in the apparatus (solution from *A* was placed in I, from *B* in II, and from *C* in III). This gave 48 millivolts,[8] III being negative to I in the external circuit (hence

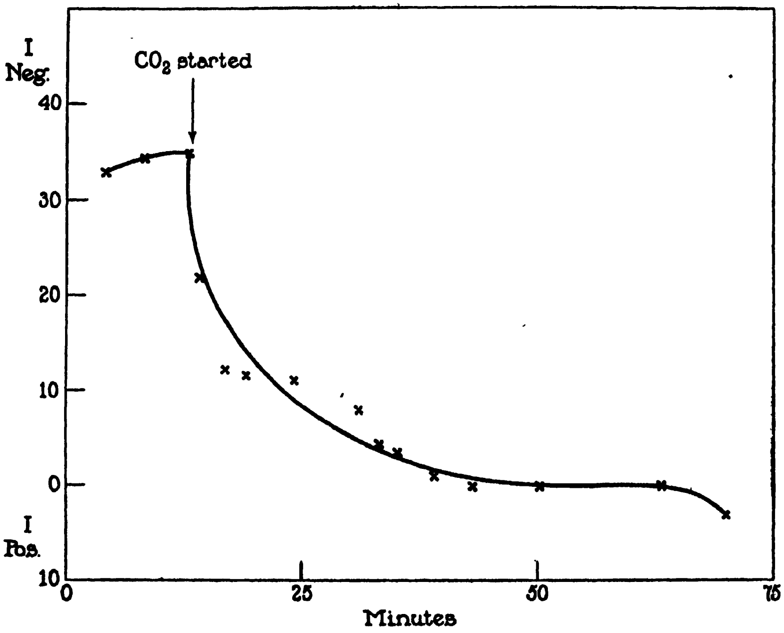


FIG. 2. At the start liquid from *A* in the model is placed in I (Fig. 1) and is 33 millivolts negative (in the external circuit) to 0.001 M $KHCO_3$ which is placed in III. Liquid from *B* is placed in II. When CO_2 is bubbled through III it reacts with K*G* in *B* to form $KHCO_3$ which dissolves in *C* and reverses the P.D. in the course of 70 minutes.

---

[8] This is the average of 5 determinations giving 48, 49, 51, 37, and 53 respectively.

in the model C is negative to A). Enough KCl[9] was then added to I to make the concentration of potassium equal to that in III and the reading became practically zero[10] despite the fact that I was more alkaline than III (this will be discussed later).

The measurements were made by means of a Compton electrometer (Cambridge Instrument Co.) the use of which was kindly loaned by Dr. Irwin. The shielding and grounding were so carefully carried out that steady readings could be made even with extremely high resistances in the circuit.

Unless the measurements became fairly steady after the first 2 minutes the experiment was rejected (a drift of more than 10 per cent between the 2nd and 10th minute not being acceptable).

It is of interest to know whether the negativity of C is due to increase of K^+ or to decrease of H^+. At the start the activity of H^+ in C was between 10^{-5} and 10^{-6} but at the steady state it was $10^{-7.5}$: hence there is about a hundredfold decrease which might account for the change in potential of (45 + 48 =) 93 millivolts were it not for the fact that the activity of K^+ in the meantime increased from zero to (0.63) (0.63)[11] = 0.40.

In the presence of so much K^+ it would seem that these variations in H^+ do not cause much change of potential as was indicated by the fact that when the concentration of potassium in I and III was equalized (as just described) the P.D. was practically zero despite the difference in pH value.

In order to test this further a solution of 0.6 M KCl was placed in I and in III: in II was placed G.C. mixture shaken with 0.6 M KCl. As expected this gave zero. Enough HCl was now added to III to make the difference of pH value on the two sides more than 2 pH units; *i.e.*, greater than in the model (in the model the pH value[12] of A is 9.1 and that of C is 7.5, a difference of 1.6). The reading was still zero or so near it as to be within the limits of experimental error.[13]

---

[9] KCl has about the same effect on potential as $KHCO_3$.

[10] Repeated four times.

[11] The activity coefficient 0.63 is obtained by interpolation from the data of Harned (Harned, H. S., *J. Am. Chem. Soc.*, 1929, **51**, 416) on the assumption that it is the same as in a solution of 0.63 M KCl and that the activities of K^+ and Cl^- in such a solution are equal.

[12] The pH values where determined colorimetrically as described in a former paper.[2]

[13] Repeated three times.

It is therefore clear that in the presence of so much K^+ the observed variations in pH value are negligible as far as effect on potentials is concerned.

We may therefore conclude that in the model the penetration of potassium creates an outwardly directed potential against which it continues to diffuse inward, thereby increasing the outward potential. This continues until the steady state is reached.

It is evident that this potential may be due to phase boundary potential or to diffusion potential.[14] In the latter case we must take into account the inward diffusion of KG, creating an inwardly directed potential (due to the greater mobility[15] of K^+ as compared with G^-) and the outward diffusion of $KHCO_3$, producing an outwardly directed potential (due to the greater mobility[15] of K^+ as compared with HCO_3^-).

In the case of living cells a similar situation might exist. It has been suggested[1,2] that potassium may form in the non-aqueous layers of the protoplasm a compound, KX, which (like KG in the model) reacts in the sap with an acid HA to form KA: subsequently HA is exchanged for HCl existing in the external solution. If in the non-aqueous layers of the protoplasm KX and HCl pass inward practically undissociated, and KCl outward, more slowly[16] but with a higher degree of dissociation, the net movement of potassium ions and of chloride ions in the non-aqueous layer will be an outward one so that there might be an outwardly directed diffusion potential[17] due to KCl. This might account in part for the outwardly directed potential observed in *Nitella* when the sap contains 0.025 M KCl + 0.025 M NaCl and[18] the outside of the protoplasm is in contact with tap water. (The higher concentration of KCl inside the cell would be due to the energy of metabolism.)

---

[14] Donnan potential may be ruled out because there are no ions which cannot pass through the non-aqueous phase.[2]

[15] This is indicated by measurements of the concentration effect.

[16] Just as in the model $KHCO_3$ passes outward more slowly than KG passes inward. It is supposed that KG is very little dissociated in the G. C. mixture.[2]

[17] This would be aided by the higher concentration of KCl and possibly by a greater difference between the mobility of anions and cations in the non-aqueous layers.

[18] Since the protoplasmic surfaces are not identical an organic electrolyte formed between these surfaces may set up an outwardly directed potential which is greater than the corresponding inwardly directed potential. This would account for the outwardly directed potential observed when sap is placed on both sides of the protoplasm (*cf.* Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, **11**, 391).

It is of interest to note that if we regard the P.D. as due to diffusion potential we may say that in the model, as well as in *Valonia*, the mobility of the cations appears to be greater than that of the corresponding anions.

SUMMARY

In a model consisting of a non-aqueous layer (representing the protoplasm) placed between an inner, more acid, aqueous layer (representing the sap) and an outer, more alkaline, aqueous solution (representing the external solution bathing a living cell) the penetration of potassium creates an outwardly directed potential against which potassium continues to diffuse inward, thereby increasing the outward potential. This continues until the steady state is reached. The potassium sets up less potential in entering than in escaping and the net result is an outwardly directed potential. A similar process appears to take place in certain living cells.

[Reprinted from BIOCHEMISCHE ZEITSCHRIFT, July 19, 1932, Vol. **250**, Nos. 1-6,
pp. 564–567]

EIN REDUKTIONS-INDIKATOR IM POTENTIALBEREICH DER WASSERSTOFFÜBERSPANNUNG

VON L. MICHAELIS

(*Aus den Laboratorien des Rockefeller Institute for Medical Research*)

(Eingegangen am 11. Juni 1932)

Keiner der bisher bekannten reversibel reduzierbaren und oxydierbaren Farbstoffe hat ein Normalpotential, welches genügend negativ wäre, um die Reduktionsintensität von Zellen unter anaeroben Bedingungen zu messen. In dieser Mitteilung soll der Versuch gemacht werden, diese Lücke auszufüllen, unter Benutzung einer noch wenig bekannten Farbstoffgruppe, von der zunächst *ein* Repräsentant beschrieben werden soll. Zum Unterschied von den anderen Indikatoren wird hier eine farblose Substanz durch die Reduktion in einen Farbstoff übergeführt, während sonst der Farbstoff durch die Reduktion entfärbt wird.

Das Ausgangsmaterial ist $\gamma\gamma'$-Dipyridyl. Dieses wird am einfachsten nach *Dimroth* und *Heene*[1] durch Oxydation des von diesen Autoren beschriebenen Diacetyl- [tetrahydro-$\gamma\gamma'$-dipyridyl] dargestellt. Die Herstellung dieser Substanz nach *Dimroth* und *Heene* macht keine Schwierigkeit, ihre Oxydation dagegen ist ein etwas launischer Prozeß. Ich bevorzugte Mangandioxyd als Oxydationsmittel, und mitunter sind die Ausbeuten an $\gamma\gamma'$-Dipyridyl ausgezeichnet. Nachdem das Interesse für diese Substanz geweckt ist, wird es leicht sein, das Verfahren zu verbessern.

Wenn $\gamma\gamma'$-Dipyridyl in leicht saurer Lösung mit Chromchlorür reduziert wird, entsteht ein tiefvioletter Farbstoff, welcher nach *Dimroth* und *Frister*[2] das Reduktionsniveau eines Chinhydrons hat. Der Farbstoff hat eine scharfe Bande mit dem Maximum um 597 $\mu\mu$. Benutzt man ein noch stärkeres Reduktionsmittel, Zinkstaub, so geht die Reduktion über diese violette Stufe weiter zu einer farblosen

---

[1] Ber. **54**, 2984, 1921.

[2] Ber. **55**, 3693, 1922.

Stufe. Die erste Stufe der Reduktion ist reversibel, die zweite irreversibel.

Wenn man versucht, die Reduktion des $\gamma\gamma'$-Dipyridyls potentiometrisch zu verfolgen, so erhält man keine stabilen Potentiale, offenbar, weil das Potentialbereich im Gebiet der Wasserstoffüberspannung liegt. Darum wird Dipyridyl auch nicht durch Wasserstoff + Palladium reduziert. In alkalischer Lösung ist es nicht möglich, den violetten Farbstoff zu erhalten. Daraus darf man schließen, daß dieser, an sich schon nicht sehr haltbare, violette Farbstoff nur in Form des Kations einigermaßen lebensfähig ist und als freie Base irreversibel sofort zerfällt. Wenn diese Anschauung richtig ist, so muß es gelingen, einen alkalibeständigen Farbstoff zu erhalten, wenn man statt des Dipyridyls selbst eine quaternäre Base desselben benutzt. Diese sind ja so starke Basen, daß sie selbst in alkalischer Lösung noch ionisiert sind. Einige Repräsentanten dieser Körperklasse sind von *Emmert* und *Varenkamp*[1] beschrieben worden. Da die Substanz für biologische Zwecke benutzt werden sollte, bemühte ich mich, sie als Salz eines möglichst indifferenten Anions herzustellen und erhielt das Dimethyl-$\gamma\gamma'$-dipyridyliumchlorid in folgender Weise.

0,5 g $\gamma\gamma'$-Dipyridyl werden mit 3 ccm Dimethylsulfat kurz erhitzt, bis alles in Lösung gegangen ist. Nach dem Abkühlen nimmt man die getrübte Lösung mit Wasser auf, entfernt das überschüssige Dimethylsulfat mit Äther und fällt das in der wässerigen Lösung befindliche Methylsulfonat der Base mit Pikrinsäure. Das schön kristallisierte Pikrat wird abgesaugt und in 200 ccm Wasser + 50 ccm konz. HCl bis zur Lösung erhitzt, dann noch warm mit Toluol und weiterhin wiederholt mit Äther ausgeschüttelt, bis alle Pikrinsäure entfernt ist. Die Lösung wird abgedampft und der Rückstand in Methylalkohol gelöst, Benzol zugesetzt und auf dem Dampfbad so viel Methylalkohol verjagt, bis das Chlorid sich abzuscheiden beginnt. Es ist zuerst ölig und kristallisiert dann in dicken Brocken. Diese werden umkristallisiert, indem man sie in Äthylalkohol löst und eine passende Menge Benzol zusetzt. Das Salz kristallisiert in farblosen, seidenglänzenden Nadeln aus. Die Analyse (Dr. *Elek*) ergab: C 55,11%; H 5,70%; N 11,07%; Cl 26,59%. Berechnet für $C_{12}H_{14}N_2Cl_2$: C 56,25%; H 5,47%; N 10,94%; Cl 27,34%.

Wir möchten diese Körperklasse als *Viologene* bezeichnen und haben die Darstellung des Methylviologens beschrieben.

---

[1] Ber. **55**, 2322, 1922.

Der Erwartung gemäß gab diese Substanz bei der Reduktion den Farbstoff nicht nur in saurer, sondern selbst in ganz stark alkalischer Lösung. Als Reduktionsmittel kann man in alkalischer Lösung Natriumhydrosulfit ($Na_2S_2O_4$) benutzen. Die Farbe ist tiefblau und hat ihre Bande um 614 $\mu\mu$.

Wenn Methylviologen in alkalischer Lösung potentiometrisch mit Hydrolsulfit titriert wird, so erhält man gut reproduzierbare Potentialkurven, die Potentiale sind recht stabil, wenn auch nicht auf beliebig lange Zeit. Die Analyse der Titrationskurve, welche an anderer Stelle zusammen mit *E. S. Hill* beschrieben werden wird, ergab, daß das Normalpotential (Potential bei 50% Reduktion) unabhängig vom p_H ist (wie z. B. das des Systems Ferricyanid—Ferrocyanid) und $-0{,}440$ Volt bei 30°C, bezogen auf die Normalwasserstoffelektrode, beträgt. Die Titrationen konnten ebensogut bei p_H 8 wie 10 oder 13 ausgeführt werden. Bei saurer Reaktion ist es nicht möglich, vollständige Kurven zu erhalten, weil hier das Potentialbereich des Farbstoffs das Wasserstoffpotential überschneidet. Aber man darf extrapolieren, daß das Normalpotential bei saurer Reaktion das gleiche ist. Bei p_H 7 ist für 30°C das Wasserstoffpotential $= -0{,}42$ Volt; das des Farbstoffs ($-0{,}44$ Volt) ist daher schon etwas negativer, und bei z. B. $p_H = 4$ ist das Farbstoffpotential um 0,24 Volt negativer als das des Wasserstoffs.

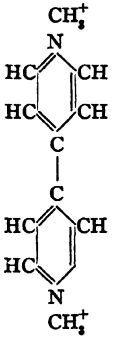
Eine Erklärung für die Unabhängigkeit des Potentials vom p_H wird auf folgende Weise gefunden. Die Analyse der Titrationskurve zeigt, daß der Farbstoff sich von dem farblosen Viologen um ein einziges Oxydationsäquivalent unterscheidet und daß er die gleiche Molekulargröße hat. Es ist also kein Merichinon im Sinne von *Willstätter* und *Piccard*,[1] sondern ein radikalartiges Semichinon. Unter Benutzung der von *Weitz* und *Fischer*[2] auf ebullioskopischem, von mir[3] auf potentiometrischem Wege begründeten Anschauungen können wir folgende Formulierung aufstellen.

---

[1] Ber. **41**, 1458, 1908.

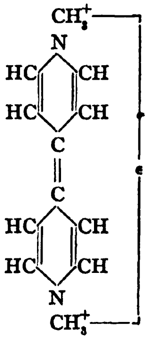
[2] Ber. **59**, 432, 1926.

[3] J. Amer. Chem. Soc. **53**, 2953, 1931.



I.

Ion der Ammoniumbase (zweiwertiges Kation).



II.

Ion des Farbstoffs (einwertiges Kation).

Unter ϵ ist ein Elektron gemeint, welches oszillatorisch abwechselnd bald dem einen, bald dem anderen Stickstoff angehört. Die äußere Elektronenschale desjenigen N-Atoms, bei welchem im gegebenen Moment das Elektron fehlt, ist ein Septett; abwechselnd hat das eine und das andere N-Atom dieses Septett oder eine Lücke im Oktett. Man braucht sich nicht vorzustellen, daß das Elektron eine so große Strecke frei durch den Raum oszilliert. Besser stellt man sich die Situation so vor, daß das Septett ein Elektron von dem benachbarten C-Atom einsaugt usf., bis das andere N-Atom ein Septett hat, und nun kehrt sich der Prozeß um. Es ist mehr als verführerisch, diese Oszillation mit dem Bandenspektrum in Zusammenhang zu bringen.

Nach diesem Bilde unterscheidet sich die oxydierte und reduzierte Form nur um ein Elektron, nicht um ein ganzes H-Atom. Darum muß das Normalpotential vom p_H unabhängig sein. Beim nicht methylierten, einfachen $\gamma\gamma'$-Dipyridyl gilt die gleiche Überlegung, wenn man CH_3^+ durch H^+ ersetzt. Eine solche Molekelart existiert aber nur in saurer Lösung, wenn das Dipyridyl ionisiert ist. Dies erklärt den Unterschied der einfachen Base von der methylierten.

[Reprinted from The JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, October, 1932,
Vol. 54, No. 10, pp. 4077–4085]

COMPLEX TYPES INVOLVED IN THE CATALYTIC OXIDATION OF THIOL ACIDS

BY MAXWELL SCHUBERT

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 11, 1932)

The study of the chemistry of the heavy metal complexes of thiol acids which in previous work[1] has been concerned with thioglycolic acid and cysteine and their compounds with cobalt has now been extended to include complexes of iron and of a thiol compound with no free carboxyl group, that is, thioglycolic ester. This extension makes possible a more detailed and clearer view of the probable mechanism of the catalytic oxidation of thiol acids to disulfide acids in the presence of iron salts by oxygen, noticed by Mathews and Walker and by Warburg and Sakuma.

The iron complexes are of particular interest as being the actual intermediate compounds involved in this catalytic process. The reaction is best shown by the addition of a trace of iron salt to a solution of thioglycolic acid or cysteine at a *P*H of 8 to 9. A red color develops with the thioglycolic acid while a violet color appears in the case of cysteine. When the dissolved oxygen has been used up this color gradually fades. Simultaneously some of the thiol acid is transformed to disulfide, but as long as there remains any thiol acid the color may be regenerated by shaking the mixture with air. A time finally comes when this color can no longer be regenerated because the thiol acid has been completely oxidized to the disulfide.

There have been two attempts to work out the mechanism of this catalytic process in detailed steps, the first by Michaelis,[2] the second by Cannan and Richardson.[3] Both theories were based on measurements made on solutions of the complexes, of such physico-chemical properties as reduction potentials, reduction capacity and reaction kinetics. In each case the catalysis was imagined as occurring in three steps: first, a formation of a ferrous complex of the thiol acid; second, an oxidation of this complex by means of oxygen to a ferric complex, which is the cause of the red or violet color observed in the catalytic process; third, an autoreduction of this ferric complex in which the iron is reduced to a divalent condition while the thiol sulfur is oxidized to disulfide with simultaneous breaking up of the complex and fading of the color.

---

[1] THIS JOURNAL, **52,** 4418 (1930); **53,** 3851 (1931).

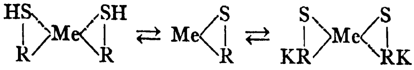
[2] L. Michaelis, *J. Biol. Chem.*, **84,** 777 (1929).

[3] Cannan and Richardson, *Biochem. J.*, **23,** 1242 (1929).

The theory that will be presented in the present paper is similar in outline but differs in the constitution of the complexes assumed as intermediate steps and attempts to present the whole picture in greater detail. The evidence is based on the types of complex actually isolated in crystalline condition from solutions containing iron and thiol acid in which the catalysis could occur if exposed to the air and from similar solutions containing cobalt in place of iron in which no catalysis can occur.

The constitution and relations of the iron complexes can be brought out most clearly by a description of an actual experiment. If an air-free solution of ferrous iron and twice as many mols of thioglycolic acid be mixed in an atmosphere of hydrogen and air-free potassium hydroxide be added drop by drop, there soon develops a yellow color. On further slow addition a yellow crystalline precipitate appears. This has the composition $Fe(SCH_2COO) \cdot 1.5H_2O$. As the potassium hydroxide addition is continued the yellow precipitate dissolves, giving a deep orange to orange-red solution. From this solution a crystalline product has been isolated having the composition $Fe(SCH_2COOK)_2$. The *P*H at this stage is in the veronal range or about 9, as will be shown in the experimental section. Finally, if enough potassium hydroxide be added, ferrous hydroxide precipitates and the orange-red color disappears. The same sequence of changes occurs when thioglycolic acid is replaced by cysteine or when iron is replaced by cobalt, the colors being, of course, different. In all these changes the metal always remains divalent as air is rigorously excluded.

This series of changes can be formulated as follows. After enough alkali has been added to bring about ionization of the carboxyl group of the thiol acid and before the appearance of the first yellow color in the solution, it is probable that the normal, and to a certain extent complex, salt I is formed. Further addition of alkali causes ionization of



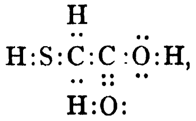
*P*H < 4 *P*H 4–6 *P*H 8–10

I II III

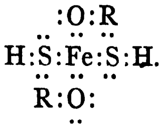
the thiol group to begin and this is facilitated by the tendency of the iron to combine with sulfur. The result is the precipitation of II in which the groups responsible for solubility are not free. That this compound should dissolve on further addition of alkali is very striking. The only way this can be made understandable is to take account of the further ionization of the thiol group and the preference of the iron to combine with sulfur rather than with oxygen. The deep orange-red solution which forms about *P*H 9 then must contain the complex in the form III. I and III differ in the distribution of primary and secondary valence bonds and II is the transition state between them, separable because of its insolubility.

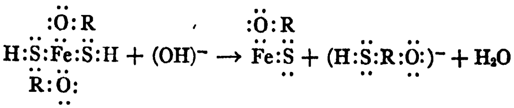
This is a point of considerable theoretical importance from the point of view of the structure of these complexes and a little further consideration will be required to see if it has any meaning at all because of the general opinion that in complex ions there is no distinction between primary and secondary valence links. In the case of symmetrical complex ions such as ammonium or ferrocyanide, there seems to be no difference between primary and secondary bonds. This probably means that the electron pair between the central atom and each coördinating group is held similarly and the electric charge of the ion is not localized at any particular hydrogen or cyanide group but is distributed over the ion as a whole.

On the octet scheme thioglycolic acid is written



$$\begin{array}{ccccccccccc} & & & & \mathrm{H} & & & & & & \\ & & & & \cdot\cdot & & & & \cdot\cdot & & \\ \mathrm{H} & : & \mathrm{S} & : & \mathrm{C} & : & \mathrm{C} & : & \mathrm{O} & : & \mathrm{H}, \\ & & & & \cdot\cdot & & :: & & \cdot\cdot & & \\ & & & & \mathrm{H} & : & \mathrm{O} & : & & & \end{array}$$

which for our purpose may be simplified to $\mathrm{H}:\underset{\cdot\cdot}{\overset{\cdot\cdot}{\mathrm{S}}}:\mathrm{R}:\underset{\cdot\cdot}{\overset{\cdot\cdot}{\mathrm{O}}}:\mathrm{H}$. The hydrogen of the carboxyl group ionizes more readily than that of the thiol group so that on dissolving iron in the thioglycolic acid the salt formed would have the structure $H:\underset{\cdot\cdot}{\ddot{S}}:R:\underset{\cdot\cdot}{\ddot{O}}:\underset{\cdot\cdot}{Fe}:\underset{\cdot\cdot}{\ddot{O}}:R:\underset{\cdot\cdot}{\ddot{S}}:H$ or, since it is at least to a certain extent complex, (A)  $\begin{array}{l} \quad :O:R \\ H:\underset{\cdot\cdot}{\ddot{S}}:\underset{\cdot\cdot}{\ddot{Fe}}:\underset{\cdot\cdot}{\ddot{S}}:H \\ \quad R:\underset{\cdot\cdot}{O}: \end{array}$. This corresponds to the complex I, but the electronic formula A shows no difference between primary and secondary bonds. In this condition the thiol group must be un-ionized; otherwise there is no reason why on making more alkaline the complex II should precipitate. The formation of II is perfectly comprehensible, however, if in the first step of the ionization of A, a dibasic complex acid, the resulting more negative character of one of the sulfur atoms makes possible a closer linkage of the iron and this sulfur atom. The preference of the iron for this closer link with the sulfur rather than its link with the oxygen is the cause of what has been called the shift of the main valence bond from oxygen to sulfur in the change of I to II. This can be represented thus

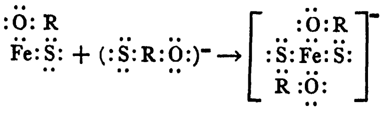


$$\begin{array}{l} \quad :\ddot{O}:R \\ H:\underset{\cdot\cdot}{\ddot{S}}:\underset{\cdot\cdot}{\ddot{Fe}}:\ddot{S}:H \\ \quad R:\underset{\cdot\cdot}{\ddot{O}}: \end{array} + (OH)^- \longrightarrow \begin{array}{l} :\ddot{O}:R \\ \ddot{Fe}:\underset{\cdot\cdot}{\ddot{S}} \end{array} + (H:\underset{\cdot\cdot}{\ddot{S}}:R:\underset{\cdot\cdot}{\ddot{O}}:)^- + H_2O$$

Now on further making alkaline the reaction

$$(H:\underset{\cdot\cdot}{\ddot{S}}:R:\underset{\cdot\cdot}{\ddot{O}}:)^- + (OH)^- \longrightarrow (:\underset{\cdot\cdot}{\ddot{S}}:R:\underset{\cdot\cdot}{\ddot{O}}:)^- + H_2O$$

is favored and again the iron can exert its preference for the link with sulfur



(B)

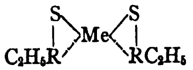
$$\begin{array}{l} :\ddot{O}:R \\ \ddot{Fe}:\underset{\cdot\cdot}{\ddot{S}}: \end{array} + (:\underset{\cdot\cdot}{\ddot{S}}:R:\underset{\cdot\cdot}{\ddot{O}}:)^- \longrightarrow \left[\begin{array}{l} \quad\quad :\ddot{O}:R \\ :\underset{\cdot\cdot}{\ddot{S}}:\ddot{Fe}:\ddot{S}: \\ \quad \ddot{R}:\underset{\cdot\cdot}{\ddot{O}}: \end{array}\right]^- \qquad (B)$$

But although the electronic formula B looks like A after complete ionization, it must be understood that a great change has taken place in the way the electron pairs between the sulfur atoms and the iron are held. Physically this is reflected in the great development of color on changing from A to B which could not be explained merely as a result of ionization.

This brings out a defect of both the valence method of representing the structure of these complexes as exemplified in I, II and III, which emphasizes the difference in the kind of bond, and the electronic method shown in A and B in which there is no difference at all. Actually the difference might be represented roughly by showing the electron pair between iron and sulfur displaced at various distances between these elements as the electronegative character of sulfur changes by ionization. It would seem likely that as the sulfur becomes more negative the electron pair would be pulled further toward the iron. It is only in some such sense as this that the distinction between primary and secondary valence bonds is to be understood in this paper.

This apparent preference of iron to link as closely as possible with sulfur is hindered when the sulfur is already covalently linked with hydrogen, but here the hydrogen is easily removable by ionization. If instead of hydrogen a benzyl group be substituted which is not displaced as the hydrogen, then the formation of more deeply colored complexes is completely hindered. The only compound that could be isolated with the S-benzylcysteine is a pink salt of the composition $Co(OOCCHNH_2CH_2SCH_2C_6G_5)_2$. The formation of the yellow or green complexes like those represented by III does not occur at all.

But if the hydrogen of the carboxyl group be replaced by an alkyl group as in thioglycolic ester then the formation of the yellow and green complexes does occur and in these the main valence links must be between metal and sulfur as



IV

These complexes of both iron and cobalt have been isolated. This is the real reason why the complex A has been identified with I where the main valence link is with the carboxyl group as in the S-benzylcysteine compound with cobalt and B has been identified with III where the main valence link is with sulfur as in IV.

The conditions in solution under which complexes of type III are formed are the same as those in which iron acts as a catalyst for the oxidation of thiol acids in the presence of air. If solutions containing III be prepared under hydrogen with careful exclusion of all oxygen, they can be kept indefinitely. But as soon as they are exposed to the air the color changes, for example, in the case of iron and thioglycolic acid from a red-orange to a much more intense dark red and almost immediately ferric hydroxide begins to precipitate while the color gradually fades. It is at this point that, in attempting to follow the chemistry of these changes made so difficult by the lability of the oxidized iron complexes, the corresponding cobalt complexes are studied instead. Cobalt complexes of the structure II and III have been described previously. The cobalto-bis-cysteinate of structure III is an intense blue-green. On exposure to the air it is oxidized like the iron complex and turns to an olive-brown but this olive-brown cobalt complex, unlike the corresponding intensely red oxidized iron complex, is stable.

Let us now consider the structure of these oxidized cobalt complexes which from their close connection with the unstable iron complexes may be expected to give us some information on the structure of these catalytically important compounds.

Kendall and Holst[4] studied the oxidation of cobalto-bis-cysteinate to cobalti-bis-cysteinate and came to the conclusion that there is an intermediate formation of a cobalti-tris-cysteinate according to the scheme

$$3\mathrm{Co^{II}(Cy)_2} \rightarrow 2\mathrm{Co^{III}(Cy)_3} + \mathrm{Co^{++}} \rightarrow 2\mathrm{Co^{III}(Cy)_2} + \text{Cystine} \qquad (1)$$

The maximum theoretical yield of cobalti-bis-cysteinate calculated on the basis of the cysteine hydrochloride used would then be 67% of the yield to be expected from a simple direct oxidation of the character

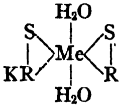
$$3\mathrm{Co^{II}(Cy)_2} \rightarrow 3\mathrm{Co^{III}(Cy)_2} \qquad (2)$$

Since actual practical yields of 75 to 80% calculated on the basis of (2) have been obtained with both cysteine and thioglycolic acid, the more complicated reaction (1) is not only unnecessary but impossible.

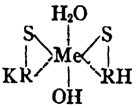
The oxidation of the cobalto-bis-cysteinate according to (2) can be imagined in three ways, giving rise to VA, VB and VC. Now on changing acidity the complex nucleus of VA being a monovalent anion can give rise to only a single potassium salt besides the corresponding free acid; that of VB being a divalent acid ion can form only two potassium salts, the free acid and a hydrochloride of the coördinated hydroxo group, while VC having a tetrabasic acid ion could give rise to at least three different potassium salts as well as the free acid and no chloride is possible.

---

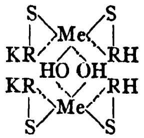
[4] Kendall and Holst, *J. Biol. Chem.*, **91**, 435 (1931).



VA



VB



VC

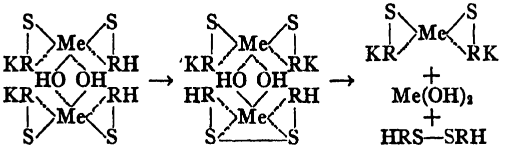
Working with both cysteine and thioglycolic acid, three salts have been obtained, a primary, a secondary and a quaternary, as well as the free acid. Although this complete series has not been prepared for either the cysteine or thioglycolic acid complexes, this is only a question of difficulty of obtaining them in crystalline form as their behavior on changing *P*H is identical, this identity of the complex nucleus being further borne out by the close similarity of their absorption spectra. The impossibility of obtaining a chloride even in strongly acid solutions of the cobalti-bis-thioglycolate contributes to discredit VB. The existence of a hydrochloride of the corresponding cysteine complex has been previously shown to be due to the amino groups present.

This chemical evidence then definitely favors the formula VC to represent the structure of the cobalti-bis-thiol acid complexes. The free acid behaves as a fairly strong acid, not being precipitable by acetic acid but requiring hydrochloric acid. On the other hand, the secondary potassium salt is the form isolated at *P*H 9 to 10. This would mean that the *pKa* values of the last two of the acid hydrogen atoms must be quite high. In a dilute aqueous solution any one of these forms of VC would be expected to be in equilibrium with several of the others. This might account for the impossibility experienced previously of determining the molecular weight of VC by cryoscopic methods.

As a result of this work the most probable course of the catalytic process seems to be first the formation of the ferrous complex III.

This is oxidized by oxygen, giving the ferric complex VC as the second step. The third step consists in a reduction of this ferric complex with a splitting off of the oxidized disulfide acid. This might be due to the generally less stable nature of ferric complexes and the tendency of these to pass into ferrous complexes. A theoretical reason for the greater stability of the ferrous complexes is suggested by Sidgwick's[5] considerations on the electron configurations of metal atoms in complexes. This theory also accounts for the great stability of cobaltic complexes. The theory is, however, open to so many objections and exceptions that although it fits the present case quite well, it cannot be taken too seriously and is mentioned merely as the only available suggestion as to why the ferric complexes undergo the third step completing the catalytic process while the cobaltic complexes stop at the second step and consequently cannot form part of a catalytic cycle.

Only one possible way of representing this third step need be mentioned.



One of the iron atoms pulls in an electron from each of the sulfur atoms with which it is combined and passes one on to the other iron atom giving, after breaking up the naturally unstable intermediate, a molecule of ferrous bis-thiol acid, one of disulfide acid and one of ferrous hydroxide, which combines with thiol acid again, completing the catalytic cycle.

For the preparation of any of the iron complexes it is necessary to carry out the whole procedure in the absence of air. This is accomplished by use of the apparatus, a diagram of which has already been published,[6] and to which reference will be made in what follows. The test-tube A is omitted; in B is placed either a freshly prepared solution of ferrous chloride or better still a pure grade of iron powder with hydrochloric acid; the flask C contains the cysteine or thioglycolic acid solution and the flask I contains the alkali necessary, which can be added drop by drop. Otherwise the manipulation is as previously described and need not be repeated here.

---

[5] Sidgwick, *J. Chem. Soc.*, **123**, 725 (1923); also Mann, *ibid.*, 651 (1929).

[6] THIS JOURNAL, **53**, 3858 (1931).

The complexes of type II are prepared as follows. After complete removal of air the ferrous chloride solution in B, containing about 20 to 25 millimoles of iron, is tipped into C containing from 25 to 50 millimoles of thiol acid. Potassium hydroxide solution is forced back into C from I drop by drop until a permanent light yellow color develops. In the case of thioglycolic acid precipitation of the complex is almost immediate and alkali may be added, while shaking the whole, until this reaches a maximum, but in the case of cysteine the precipitation is slower and it is best to proceed more cautiously. In either case the precipitated compound is light yellow and by warming slightly with a beaker of hot water crystallization is rapid. Filtration and washing are as previously described. The *P*H at which this precipitation occurs is probably about 5 as filtrates tested were acid to litmus and alkaline to congo red. After drying over sulfuric acid at less than 1 mm. pressure, these analytical results were obtained.

Anal. Calcd. for $Fe(OOCCHS)\cdot 1.5H_2O$. Fe, 32.35; S, 18.50. Found for different preparations: Fe, 32.89, 32.98; S, 18.81, 19.10. Calcd. for $Fe(OOCCHNH_2CHS)\cdot 1.5H_2O$: Fe, 27.70; N, 6.93; S, 15.84. Found: Fe, 27.89; N, 6.44; S, 16.42.

These complexes after drying are perfectly stable in air. They undergo no apparent change when suspended in water, in which they are quite insoluble. Addition of dilute alkali causes no change but if a little thio acid be added simultaneously in the absence of air, then the deep red color of the complexes III is produced.

The complexes of type III at first seemed much more difficult to prepare. This is due to the great solubility of these complexes, together with their great sensitiveness to oxidation by oxygen so that rigid exclusion of air is essential until the crystalline product is separated from solution and washed.

The potassium salt of this complex has been prepared by a long complicated process involving a preparation of ferrous hydroxide, a thorough washing of this material followed by addition of thioglycolic acid and potassium hydroxide and evaporation of the resulting solution to small volume, an addition of air-free alcohol and a cooling on ice for some twelve hours. The resulting yellow rectangular platelets are filtered and washed with alcohol. This entire set of operations is carried out in closed vessels through which a continuous stream of purified hydrogen is passed. After drying *in vacuo* over sulfuric acid the analysis agreed reasonably, considering the impossibility of any purification procedures, with $Fe(SCH_2COOK)_2\cdot 2H_2O$.

Anal. Calcd.: Fe, 16.87; S, 19.27; K, 23.50. Found, for independent preparations: Fe, 19.42, 16.25; S, 19.26, 20.00; K, 25.14, 25.87.

The sodium salt of this complex can be prepared more easily. In the test-tube B of the special flask set-up are placed 5.5 g. of $FeSO_4\cdot(NH_4)_2SO_4\cdot 6H_2O$ and 15 cc. of water and in the main flask 3 cc. of thioglycolic acid and 5 cc. of water.

After complete removal of air sodium hydroxide is slowly added from the flask I. At first the yellow precipitate of the complex II appears but this soon redissolves, giving the usual deep red solution from which, after adding 9 to 10 cc. of 7.3 *M* sodium hydroxide, there slowly crystallizes a yellow substance. It is best to shake steadily while this is in progress as the crystals tend to stick to the sides of the flask. This product is filtered, washed with air-free water and alcohol as usual. Dried *in vacuo* its analysis corresponds with $Fe(SCH_2COONa)_2 \cdot 2H_2O$. The results of two preparations are given.

Anal. Calcd.: Fe, 17.60; S, 20.11; Na, 14.46. Found: Fe, 18.46, 17.26; S, 20.45, 20.20; Na, 15.26, 15.86.

Freshly prepared and before exposure to the air the crystals are yellow and dissolve readily in air-free water, giving a raspberry-red solution which on exposure to air turns to a much deeper red. The dry crystals themselves on exposure to air turn from yellow to yellow-green and finally to an orange-red. Using a Warburg microrespiration apparatus, they were found to absorb oxygen slowly from the air. That this is not merely a case of adsorption of gas due to drying the crystals at less than 1 mm. pressure is shown by the fact that in an atmosphere of hydrogen no gas is taken up at all. In a number of experiments, making the transfer from the desiccator to the Warburg vessel as quickly as possible, which with weighing and allowing the vessel to come to temperature equilibrium in the bath occupied ten to fifteen minutes, 20 to 25 millimoles of the complex absorbed 5 to 10 millimoles of oxygen in three to four hours. About the same oxygen consumption was measured by dissolving the crystals in borate buffer. In either case an orange-red crystalline product was obtained. This product is insoluble in water, borate buffer and dilute acetic acid and when washed and dried shows an iron content of 53.0%, which with the absence of other elements makes it appear as ferric hydroxide. If dissolved in dilute hydrochloric acid and reprecipitated with alkali, the usual form of ferric hydroxide appears.

The cobalt salt of S-benzylcysteine is easily prepared by dissolving 4 g. of benzylcysteine made as described by Suter[7] in 200 cc. of hot water containing 15 g. of secondary sodium phosphate and slowly adding a mixture of 4 cc. of 1 *M* $CoCl_2$ and 25 cc. of saturated Na_2HPO_4, digesting the mixture for an hour at boiling temperature until no more cobaltous phosphate remains, filtering and washing with boiling water; 2 g. of pink crystals consisting of flat plates is obtained.

Anal. Calcd. for $Co(OOCCHNH_2CH_2SCH_2C_6H_5)_2 \cdot H_2O$: Co, 11.87; S, 12.87; N, 5.63. Found: Co, 11.69; S, 13.67; N, 5.50.

The ferrous complexes of the ethyl ester of thioglycolic acid were prepared in a manner similar to that described for complexes of type VI. For example, 7 g. of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ and 25 cc. of water are placed in test-tube B, 3.7 cc. of thioglycolic ester in flask C and after deaeration the iron is tipped into the flask C. From I 3.5 *M* potassium hydroxide is added dropwise. On the first addition of alkali a yellow precipitate forms which rapidly crystallizes but there are dark red or brown spots present which disappear for the most part on warming the mixture with a beaker of water. The yellow crystals are filtered, washed and transferred to a desiccator. It is much more difficult to get reasonable analytical data on this complex because as soon as the desiccator is opened there appears to be a rapid combination with oxygen and the dry crystalline product gets quite hot, fumes are given off and in some cases the substance even glows. This action lasts only a minute or two, after which there appears to be no further change. Analytical data on the three best preparations are as follows.

---

[7] Suter, *Z. physiol. Chem.*, **20**, 562 (1895).

Anal. Calcd. for $Fe(SCH_2COOC_2H_5)_2$: Fe, 19.05; S, 21.77. Found: Fe, 18.74, 19.58; S, 22.07, 20.60. Calcd. for $Fe(SCH_2COOC_2H_5)\cdot 2H_2O$: Fe, 16.97; S, 19.39. Found: Fe, 17.19; S, 19.57.

The same procedure was followed to prepare the corresponding cobalt complex, which separates as thin leaflets of a gold-green color with a metallic sheen. This compound after washing and drying appears to be stable in air.

Anal. Calcd. for $[Co(SCH_2COOC_2H_5)_2]_2HSCH_2COOC_2H_5$: Co. 16.52; S, 22.39. Found: Co, 16.86, 17.45; S, 23.01, 23.03.

This complex dissolves in chloroform to give a deep red solution. If this complex is refluxed with chloroform for a while, filtered and treated with an equal volume of alcohol, a gelatinous precipitate is formed which may be redissolved on gentle warming. On slow cooling a granular deposit of very tiny black crystal forms. The same substance is also formed by mixing a solution of 2.5 cc. of thioglycolic ester in 10 cc. of alcohol with one of 2.5 g. of $CoCl_2\cdot 6H_2O$ in 30 cc. of alcohol and bubbling air through the mixture for an hour. After standing for a day a fine granular deposit settles out which is washed most conveniently with alcohol by centrifuging, yielding 1 g. of product after drying. Either method of preparation gives the compound $Co(SCH_2COOC_2H_5)_3$.

Anal. Calcd.: Co, 14.18; S, 23.08. Found: Co, 14.60, 13.90; S, 23.27, 22.99.

Cobalt complexes with nuclei of type VC have already been described, the only one which is new being a tetrapotassium salt of the cysteine complex. The simplest method of preparation is to start with the corresponding free acid, 5 g. of which is dissolved in 15 cc. of 3.7 *M* potassium hydroxide; then 25 cc. more of this potassium hydroxide solution is added, followed by 50 cc. of alcohol, stirring vigorously during this addition. After standing for an hour the mixture becomes almost solid with masses of very fine hair-like crystals which are filtered off and washed with a little cold water and then with alcohol.

Anal. Calcd. for $[Co(SCH_2CHNH_2COOK)_2OH]_2$: Co, 15.05; S, 16.34; K, 19.89. Found: Co, 14.97; S, 16.47; K, 20.11.

Attempts to prepare the corresponding salt of the thioglycolate complexes resulted only in the separation of uncrystallizable liquids. The barium salts prepared at these high alkalinities were different from those made at a *P*H range of 8 to 10 but likewise resisted attempts to crystallize and so were unsuitable for analysis.

Thanks are due to Mr. Bitterlich of this Laboratory for all analytical work done in connection with this paper.

SUMMARY

By working in the absence of oxygen a number of complexes of divalent iron with cysteine, thioglycolic acid and ethyl thioglycolate have been prepared and their structures discussed in some detail. The rôle of these complexes in the catalytic oxidation of thiol acids in the presence of iron salts is pointed out and on this basis as well as that of some previous work a theory of the mechanism of this catalysis is presented.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1932,
Vol. 56, No. 4, pp. 521-530]

STUDIES ON THE QUANTITATIVE ACTION OF A SPECIFIC ENZYME IN TYPE III PNEUMOCOCCUS DERMAL INFECTION IN RABBITS

BY KENNETH GOODNER, PH.D., AND RENÉ DUBOS, PH.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, June 3, 1932)

Avery and Dubos (1-3) have reported the isolation of a bacterium capable of specifically decomposing the capsular polysaccharide of Pneumococcus Type III. This decomposition was shown to be due to the action of an intracellular enzyme which could be extracted in active form from the bacilli. Potent preparations of this enzyme protected mice against infection with virulent Type III Pneumococcus and exerted a curative action on an infection already established.

Recently we have reported that this enzyme, when used in sufficient amounts and under stated conditions, was capable of bringing about a favorable and early termination of the experimental disease induced by infecting rabbits intradermally with Type III Pneumococcus (4).

A strain of Type III Pneumococcus which was especially virulent for rabbits was used in producing the characteristic infection described in detail in previous papers (5-8). An edematous lesion makes its appearance at the point of inoculation in the skin of the rabbit's flank and rapidly spreads ventrally, creating within 15 to 20 hours a widespread lesion most pronounced in the dependent portions of the skin. With the development of the local lesion there is a rapid elevation of body temperature and this remains at high levels until death or eventual recovery. Pneumococci escape from the lesion into the general circulation and create a bacteriemia of varying but generally increasing severity. Following the intravenous injection of large quantities of enzyme 24 hours after infective inoculation, a large majority of the animals survived. The injection of the enzyme is followed immediately by the disappearance of organisms from the blood stream. The temperature at first rises but within a few hours begins to fall and normal levels are generally reached within 24 hours. With the subsidence of the bacteriemia and the fall in temperature the local lesion begins to show signs of healing and the skin eventually returns to its normal state. Successful experiments were also reported in which smaller amounts of enzyme were injected repeatedly over a period of days.

This earlier work was chiefly concerned with the qualitative aspects of the problem. However, the results indicated that large quantities of enzyme appeared to be necessary for successful therapy in rabbits which had a high bacteriemia while smaller amounts seemed adequate in animals having a lesser number of organisms in the blood at the time of treatment.

The experiments reported in the present paper deal with the quantitative factors involved in the action of the specific enzyme in this experimental disease.

EXPERIMENTAL

The general plan of these experiments was to give each infected rabbit one intravenous injection of a determined amount of enzyme 24 hours after infective inoculation. For the most part the experimental methods were the same as those reported in the previous paper (4).

Culture.—The rabbit-virulent strain of Type III Pneumococcus was maintained in rabbit blood broth with frequent animal passage. Virulence for rabbits was such that 0.000,01 cc., given intradermally, caused death or a protracted disease of severe character.

Infection.—Healthy male rabbits weighing from 1,800 to 2,000 gm. were used. Animals were injected intradermally, at a site midway on the flank area, with 0.2 cc. of a dilution of the culture containing the desired number of organisms. In this series certain of the animals received 0.001 cc. of culture; others, for reasons to be mentioned below, received 0.2 cc.

Enzyme Preparations.—The enzyme preparations used in these experiments were, for the larger part, purified and concentrated lots prepared by the method described by Dubos (9). The potency was carefully determined by the method previously described.

Enzyme Injections.—Each treated animal received a single injection of enzyme 24 hours after infective inoculation. The desired amount of the enzyme preparation was warmed to 37°C., and injected intravenously at a rate not greater than 1.5 cc. per minute.

Blood Cultures.—The number of organisms per cc. of blood was determined by a procedure previously described in detail (5). In brief, the method consists in plating 0.4 cc. of blood taken from the marginal ear vein with an accurately graduated syringe. It is obvious that this method is open to many experimental errors and that counts so obtained are not entirely accurate. An analysis of the results, however, shows that they are relatively significant.

The estimation of the number of pneumococci in the circulating blood takes no account of the number in the lesion. This cannot be readily determined. If the edema fluid is removed and cultured, the number of pneumococci present is usually found not to be extremely large. However, a large number may be present in the tissues. The number in the lesion undoubtedly varies within limits depending upon the amount of the tissue involved, and possibly upon other unknown factors.

Dubos and Avery (2) have shown by experiments *in vitro* that, after a definite incubation period, the total amount of specific substrate decomposed bears a quantitative relationship to the concentration and activity of the enzyme preparation used. If it were possible to apply this finding to experimental infections, the minimal amount of enzyme necessary to bring about recovery should bear a quantitative relationship to the amount of specific capsular polysaccharide in the animal body. This substance is of course associated with the pneumococci but may also be present in a free form entirely detached from the bacteria. It is technically impossible to determine the total amount of the specific polysaccharide in the infected animal or even to estimate the total number of pneumococci. The nearest approximation is the determination of the number of viable pneumococci in the circulating blood. Other factors being the same, the degree of blood infection should, on theoretical grounds, bear some quantitative relationship to the amount of enzyme necessary to bring about recovery. When no invasion of the blood has occurred this relationship, of course, would not hold.

As the experimental work progressed it became apparent that the amount of enzyme necessary to bring about recovery was definitely related to the number of organisms in the blood stream. It therefore became desirable to study a large number of animals showing wide variations in the degree of blood infection. With an infective amount of 0.001 cc. of culture there was considerable individual variation in the animals, the number of organisms per cc. of circulating blood varying from 5 to 10,000, the higher numbers being exceptional. In order to insure an early and massive blood invasion the infective inoculum was increased to 0.2 cc., an amount 200 times greater than that previously used. This gave rise to an infection which differed from the previous one only in that it was much more rapidly fatal if untreated and in that the numbers of pneumococci per cc. of blood were relatively enormous, frequently too great to estimate by the plate method. In the series of animals reported in this paper about one-half have been infected with 0.2 cc. and one-half with 0.001 cc. of culture.

An apparently insurmountable difficulty was that at the time of treatment there was no knowledge of the number of pneumococci in the blood stream. This information is necessarily not available until 24 hours thereafter. In the hope of finding some index of the bacteriemia which might be available at once, we have compared the number of organisms in the blood stream with the height of the temperature, the temperature trend, the leukocyte count, the trend of this count, the appearance and magnitude of the lesion, and the weight trend. No satisfactory correlation was obtained with any of these factors. Because of this fact the amounts of enzyme given have been arbitrarily chosen.

The Amount of Enzyme and the Outcome of the Disease

We have now studied a series of 85 rabbits of which 61 have been treated with a definite amount of enzyme 24 hours after infective inoculation. The others have served as controls. The data on this series of animals are shown in Table I. The animals have been arranged in groups depending on the numbers of organisms in the blood stream at the time of treatment. Thus in Group A are placed the animals in which the blood cultures were negative; in Group B, those with 3 to 10 organisms per cc.; etc. In each group the animals are arranged in series according to the amounts of enzyme each received. In each instance the degree of bacteriemia and the results of the treatment are indicated.

Group A.—Of six untreated controls, one survived. Among the treated animals there were scattering deaths until the amount of enzyme used was greater than 2.5 units. Even in these fatal cases, however, death occurred, on an average, over 4 days later than in the controls. When 3 or more units were used, all animals survived.

Group B.—In this group are included those animals in which the blood cultures showed from 3 to 10 organisms per cc. The smallest amount of enzyme used in this group was 2.5 units. All these animals survived whereas the untreated controls died.

Group C.—In the animals in this group, at the time of treatment, 11 to 100 organisms per cc. of blood were found. An apparent irregularity is caused by the fact that one animal died after receiving 10 units of enzyme whereas another survived after only 6.6 units. However, it should be noted that the bacteriemia was much higher in the former animal. The minimal amount of enzyme efficacious in this group, therefore, was between 10 and 20 units.

TABLE I

Results of the Administration of Various Amounts of Specific Enzyme in Infected Rabbits Having Varying Numbers of Type III Pneumococci per Cc. of Blood

Determined amounts of enzyme preparations injected intravenously into rabbits 24 hrs. after infective inoculation with Type III Pneumococcus

Group	Rabbit	Bacteriemia at 24 hrs.	Units of enzyme	Result
A	1	Negative	None	D 114
	2	"	"	" 88
	3	"	"	" 70
	4	"	"	" 64
	5	"	"	" 53
	6	"	"	S
	7	"	1	"
	8	"	1.25	"
	9	"	2	D 162
	10	"	2	S
	11	"	2.5	D 192
	12	"	2.5	" 190
	13	"	3	S
	14	"	3	"
	15	"	6.6	"
	16	"	10	"
	17	"	20	"
	18	"	100	"
	19	"	100	"
	20	"	100	"
B	1	10	None	D 84
	2	5	"	" 84
	3	5	"	" 51
	4	3	"	" 150
	5	3	2.5	S
	6	3	6.6	"
	7	3	10	"
	8	3	17.5	"
	9	10	20	"
	10	10	50	"
	11	5	100	"
	12	8	100	"
	13	10	100	"
	14	10	100	"
C	1	85	None	D 111
	2	78	"	" 36
	3	52	"	" 55
	4	40	"	" 52

TABLE I—*Continued*

Determined amounts of enzyme preparations injected intravenously into rabbits 24 hrs. after infective inoculation with Type III Pneumococcus

Group	Rabbit	Bacteriemia at 24 hrs.	Units of enzyme	Result
C—*concluded*	5	13	None	D 62
	6	23	3	" 40
	7	20	3	" 108
	8	15	6.6	S
	9	73	10	D 68
	10	16	20	S
	11	48	37.5	"
	12	55	50	"
	13	25	100	"
	14	20	100	"
D	1	789	None	D 60
	2	345	"	" 51
	3	210	"	" 86
	4	140	"	" 86
	5	630	4	" 150
	6	345	5	" 105
	7	315	5	" 120
	8	580	6.6	" 72
	9	103	10	" 88
	10	255	20	S
	11	265	26.6	"
	12	180	37.5	"
	13	457	100	"
	14	165	100	"
	15	157	100	"
E	1	5,040	None	D 42
	2	2,400	"	" 77
	3	3,760	4	" 102
	4	7,840	13.3	" 112
	5	5,080	17.5	" 114
	6	8,120	18	" 58
	7	6,720	20	" 60
	8	1,760	50	S
F	1	∞	None	D 39
	2	∞	"	" 40
	3	∞	"	" 38
	4	∞	1	" 40
	5	25,440	1.25	" 40

TABLE I—*Concluded*

Determined amounts of enzyme preparations injected intravenously into rabbits 24 hrs. after infective inoculation with Type III Pneumococcus

Group	Rabbit	Bacteriemia at 24 hrs	Units of enzyme	Result
F—*concluded*	6	∞	5	D 66
	7	20,880	17.5	" 140
	8	13,440	17.5	" 88
	9	∞	20	" 70
	10	∞	20	" 80
	11	∞	20	" 60
	12	37,120	100	" 30
	13	35,840	100	" 36
	14	26,580	100	" 26

S = survival of animal. D = death of animal, at indicated number of hours after infective inoculation. ∞ = number too great to estimate.

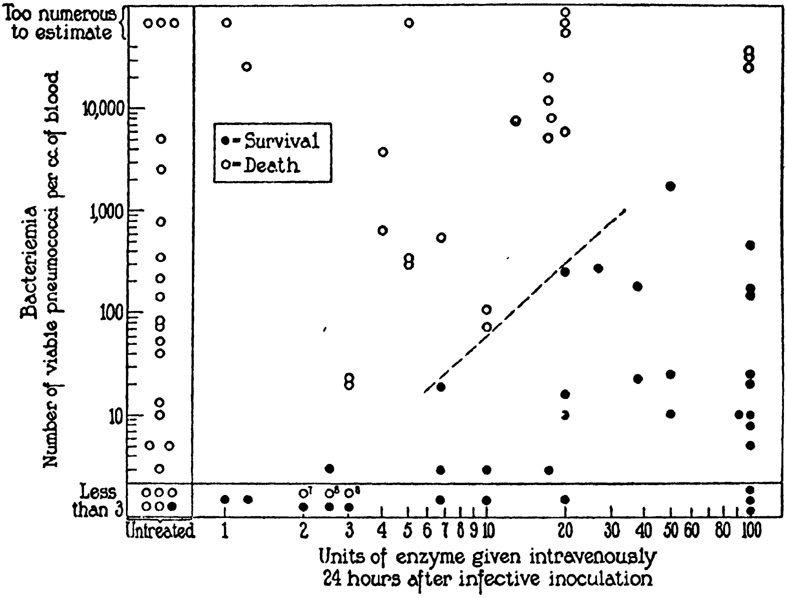
Group D.—The animals in this group showed 100 to 1,000 organisms per cc. of blood. In this group all animals which received 20 or more units of enzyme recovered. The controls died somewhat earlier than those of the preceding groups but again the average length of life of the treated animals which died was a day and a half longer than that of the controls.

Group E.—In this group are listed the animals with blood cultures showing 1,000 to 10,000 pneumococci per cc. The degree of bacteriemia in this group was very high. In spite of this the animal which received the largest amount of enzyme, 50 units, recovered. Moreover, the animals treated with smaller amounts, although they did not recover, nevertheless lived somewhat longer than did the controls.

Group F.—In these animals there were present 10,000 or more pneumococci per cc. of blood. The administration of the enzyme, even as much as 100 units, was without favorable result in these cases. Although in this series of animals receiving but a single injection none recovered, infections of this order have been successfully treated by repeated injections of large amounts of enzyme over a period of days.

Considering Table I as a whole it becomes obvious that larger amounts of enzyme are necessary as the number of organisms in the blood becomes greater. Thus 6.6 units of enzyme were sufficient to bring about recovery in an animal showing 15 organisms per cc. of blood, 20 units sufficed in an animal having 255 organisms per cc., 50 units were required in an animal with 1,760 organisms per cc.; however, a single injection of even 100 units failed to bring about recovery when the number of bacteria per cc. of blood exceeded 20,000.

These results are best illustrated by a graphic presentation as in Text-fig. 1. It will be noted that in general the symbols indicating the fatalities occupy one section of the figure whereas those which indicate the survivals occupy another. The data are not sufficient to allow of the actual plotting of a curve which might separate these areas but a broken line has been drawn which indicates roughly the two zones. The results are not sharply defined in that portion of the chart representing the animals in which the blood cultures were negative and the amounts of enzyme employed were very small. It is believed that this is due to the fact that in these animals the results depend entirely on the number of organisms found in the lesion and this may of course vary in individual animals.



TEXT-FIG. 1. Results of the administration of various amounts of specific enzyme in infected rabbits having varying numbers of Type III pneumococci per cc. of blood. A broken line indicates a suggested boundary between zones of effective and non-effective therapy. In three instances the day of death is indicated by small numerals.

It may be pointed out that, as the number of pneumococci in the blood increases, the increase in the relative amount of enzyme necessary to combat the infection becomes less.

DISCUSSION

This study has shown that the amount of enzyme necessary to bring about recovery in the infected rabbit bears a definite relationship to the number of organisms present in the blood stream at the time of treatment. Thus, in animals with negative blood cultures or with bacteriemia of low grade, an amount of enzyme as small as 5 units may save the life of the animal; with a bacteriemia of 100 to 1,000 organisms per cc. of blood, 20 units may be necessary; with a bacteriemia of 1,000 to 10,000 organisms per cc., 50 units were necessary. In animals in which the bacteriemia exceeded 10,000 organisms per cc. a single injection of even 100 units was not effectual in saving the life of the animal, although, as previously mentioned, infections of this order have been successfully treated by repeated injections of large amounts of enzyme over a period of days.

Although the quantitative relationship established in these experiments is between the amount of enzyme and the number of pneumococci present in the blood, the fundamental relationship is that between the quantity of enzyme and the total amount of specific capsular polysaccharide present in the body. The degree of bacteriemia serves merely as an index of the latter.

The enzyme is not a therapeutic substance *per se*, but one which, by decomposing the capsular substance of the pneumococci and thus preparing the bacterial cells for phagocytosis, initiates a process which the body must be in a condition to carry on if the animal is to recover. Hence, in the use of the enzyme, this capacity of the body to complete the reaction must be reckoned with.

SUMMARY

The enzyme which specifically decomposes the capsular polysaccharide of Type III Pneumococcus must be used in certain definite amounts in order to bring about the recovery of rabbits infected intradermally with this organism. The experiments reported in this paper indicate that the minimal amounts of enzyme required bear a definite relationship to the severity of the infection as gauged by the number of pneumococci present in the circulating blood.

BIBLIOGRAPHY

1. Avery, O. T., and Dubos, R., *Science*, 1930, **72**, 151.
2. Dubos, R., and Avery, O. T., *J. Exp. Med.*, 1931, **54**, 51.
3. Avery, O. T., and Dubos, R., *J. Exp. Med.*, 1931, **54**, 471.
4. Goodner, K., Dubos, R., and Avery, O. T., *J. Exp. Med.*, 1932, **55**, 393.
5. Goodner, K., *J. Exp. Med.*, 1928, **48**, 1.
6. Goodner, K., *J. Exp. Med.*, 1928, **48**, 413.
7. Rhoads, C. P., and Goodner, K., *J. Exp. Med.*, 1931, **54**, 41.
8. Goodner, K., *J. Exp. Med.*, 1931, **54**, 847.
9. Dubos, R., *J. Exp. Med.*, 1932, **55**, 377.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1932,
Vol. 56, No. 4, pp. 601–608]

INFECTIOUS MYXOMATOSIS (SANARELLI) IN PREGNANT RABBITS

BY DOUGLAS H. SPRUNT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

PLATES 32 AND 33

(Received for publication, June 13, 1932)

Viruses exhibit an intimate type of parasitism. This phenomenon is emphasized by the fact that many of the active agents multiply only in one species of host and only in certain tissues of that host. In the course of some experiments with infectious myxomatosis it was noticed that the clinical picture and morbid anatomy of the disease in pregnant rabbits are different from those in non-gravid animals. These differences indicate a line of approach to a better understanding of the host-virus complex and are sufficiently striking to be recorded.

Many investigators have observed that the course of certain tumors is influenced by pregnancy. Inasmuch as the reports and views regarding this matter are contradictory, some of the more prominent ones are cited. Morau (1), Ehrlich (2), Uhlenhuth and Weidanz (3) reported that pregnancy retards the growth of transplantable tumors in mice. Cuénot and Mercier (4), however, observed an acceleration of growth of similar tumors, while Albrecht and Hecht (5) stated that pregnancy has no effect on such neoplasms. Askanazy (6), Jentzler (7), and Herzog (8) reported that the growth of tumors in rats was accelerated by pregnancy. Kross (9), however, claimed that pregnancy has no effect on the rate of growth of neoplasms in rats.

Rous (10) in studying the effect of pregnancy on embryonic transplants, which he had shown to behave in many respects like transplantable tumors, reported the following experiments. Several loops of the uterus of a pregnant mouse were removed. A small fragment from one of the embryos was injected beneath the skin of the mother. This piece of tissue became vascularized but did not grow until the remaining part of the uterus was emptied. In other experiments he found that embryonic transplants grew equally well in non-gravid mice and in pregnant mice but that the transplants became differentiated into a larger number of tissues in the latter group of animals. Fischera (11) made similar observations concerning embryonic transplants in rats. Shattock, Seligmann, and Dudgeon (12), however, reported that gestation had no effect on the growth of transplanted fetal cartilage.

The effect of pregnancy on spontaneous tumors in animals has not been investigated to any extent. Slye (13) stated that neoplasms occur less frequently in gestating mice than in virgin mice. Krotkina (14) observed that in a rabbit with a tar cancer the neoplasm became smaller during each pregnancy. Rous (10) observed that the growth of a spontaneous tumor in one mouse was accelerated during pregnancy.

The reports concerning neoplasm in pregnant women are with a few exceptions in accord. Bainbridge (15) in an excellent review of the literature concluded that pregnancy exerts a stimulating and hence malign influence upon coexistent cancer. This he believes to be true not only of cancer of the breast and uterus where an increase in the blood supply might account for the accelerated growth but also of neoplasms in other parts of the body with the possible exceptions of certain epitheliomas.

Taliaferro (16) and his associates found that during the latter stages of pregnancy the resistance of rats to *Trypanosoma lewisi* is lowered. No other reports of experimental work dealing with the effect of pregnancy on diseases due to microorganisms have been encountered.

The mortality in acute infections is generally considered to be increased by pregnancy. This change is attributed by Williams (17) to the extra strain of the abortion which accompanies the infection. Weintraub (18) pointed out in his review of the literature on influenza that women may not die because they abort but abort because they are dying. Many authors have claimed that the susceptibility to influenza is increased by pregnancy. Weintraub (18) is of the opinion that this may be only apparent since influenza is most common in early adult life, the period during which women are most frequently pregnant. It has been maintained that the incidence of acute infections among pregnant women is lower than among the non-pregnant. With the exception of scarlet fever (19) there appears little evidence to support this hypothesis.

Methods and Materials

Virus.—The virus employed in these experiments is the strain used by Rivers (20). Its virulence may have been somewhat enhanced by numerous passages through animals. The virus was prepared by dissecting the myxomatous material, occurring at the point of an intradermal inoculation of the virus, free from skin and muscle. The infectious tissue was then ground thoroughly with alundum and diluted with sufficient distilled water to make a 10 per cent suspension. The virus was found to be active in such suspensions diluted 1.0×10^{-4} and 1.0×10^{-5}.

Animals.—Pregnant and non-pregnant female rabbits from the same source were used. The gestation period of the gravid animals had proceeded for about 20 days—the normal gestation period is about 30 days.

Inoculation.—The rabbits were inoculated intradermally. The points chosen for inoculation were well up on the rabbit's side in order that the mammary glands would not be involved. This precaution was considered necessary because the mammary tissue has an increased blood supply during pregnancy which might affect the local lesion and the dissemination of the virus. The virus was diluted with Locke's solution in multiples of 10 up to 1.0×10^{-6}, 0.25 cc. of the different dilutions being injected as indicated below.

Fixation and Stains.—Tissues used for histological study were removed from the animals within an hour after death except in the instances where it appeared that a rabbit would not live through the night. Under such conditions the animal was killed and necropsy was performed immediately. Tissues from the heart, lungs, spleen, liver, adrenals, kidney, ovaries, intestines, bone marrow (shaft of the femur), skin, lymph nodes, and brain were fixed in Zenker's fluid (5 per cent acetic acid) and in formalin (10 per cent or 3.7 per cent formaldehyde gas) and stained with hematoxylin and eosin, with Mallory's phosphotungstic acid hematoxylin, and according to the Giemsa method. Sections of the liver were stained with Sudan III for fat.

EXPERIMENTAL

Experiments were designed to ascertain whether pregnancy altered the resistance of rabbits to the virus. Hence some of the animals were given relatively small amounts of the active agent.

Experiment 1.—Each of 3 pregnant and 2 non-pregnant female rabbits received intradermal inoculations, 0.25 cc., of dilutions of the same virus emulsion ranging from 1.0×10^{-2} to 1.0×10^{-6}.

Experiment 2.—Each of 6 pregnant rabbits and 1 non-pregnant female rabbit were given 3 intradermal injections, 0.25 cc., of dilutions of the virus emulsion ranging from 10^{-4} to 10^{-6}.

Experiment 3.—Each of 4 pregnant and 8 non-pregnant female rabbits received 6 intradermal injections, 0.25 cc., of dilutions of the virus emulsion ranging from 10^{-1} to 10^{-6}.

In the 3 experiments, 13 pregnant and 11 non-pregnant female rabbits were inoculated with the virus of infectious myxomatosis and the resulting clinical picture and morbid anatomy were observed. Inasmuch as the results of the experiments were similar, they will not be described separately.

Clinical Picture in Non-Pregnant Rabbits.—On the 3rd day slightly elevated skin lesions appear at the points of inoculation. These lesions increase in size and on the 5th day secondary lesions in the skin are usually visible. The primary lesions at this time become hemorrhagic and are capped by small vesicles. The subcutaneous tissues of the eyelids, ears, and genitalia as a rule show involvement on the 6th day and by the 9th the eyes are closed. On the day of the disease on which the animals die, usually the 9th after inoculation, they are dyspneic.

Clinical Picture in Pregnant Rabbits.—The course of the disease in the pregnant animals is similar to that described above with the following exceptions. The skin lesions are less elevated. Secondary lesions of the skin and involvement of the subcutaneous tissues of the ears are frequently absent or small. Approximately half of the animals abort on the 8th or 9th day of the disease. The remainder carry the young until death which usually occurs on the 9th day. The pregnant and non-pregnant animals responded in like manner to the various dilutions of the virus.

Morbid Anatomy of Non-Pregnant Rabbits.[1] *Liver.*—Small areas of focal necrosis are seen in the liver but such lesions are not uncommon in "normal" rabbits. In 2 animals a few necrotic cells are seen around the central vein.

Skin.—The lesions are slightly elevated, ovoid, firm, purplish masses that extend through the superficial muscles into the deeper fascial layer. A cross-section reveals a myxomatous-like tissue which contains numerous small hemorrhagic areas and dilated blood vessels. Histological preparations reveal hyperplasia, necrosis, and vesiculation of the epithelium. The cytoplasmic inclusions described by Rivers (20) are seen. Beneath the epithelium are some degenerating polymorphonuclear cells. Extending throughout the subcutaneous tissue are numerous small strands of fibrin. The superficial muscle layer is atrophied and in places necrotic. Around the hair follicles, glands, and blood vessels are a number of proliferating stellate cells, characteristic of the disease.

Lungs.—Only 4 animals in this group have secondary myxomatous lesions in the lungs. The lesions first appear in the lymphoid tissue around the bronchi and are frequently infiltrated with polymorphonuclear leucocytes. From this point of origin the pathological process extends around the blood vessels and into the interstitial tissue. The epithelial cells of the bronchi in close proximity to the myxomatous tissue are hyperplastic.

Spleen.—In only a few instances are the spleens enlarged. Myxomatous secondary lesions, however, are found in the Malpighian corpuscles of all the rabbits (Fig. 1). In most of the animals only a few follicles are involved while in others only a few normal lymphoid cells remain.

Lymph Nodes.—The architecture of the lymph nodes draining the region of inoculation is destroyed. The lymphoid cells are replaced by a large number of stellate elements among which are scattered numerous red blood cells, some fibroblasts, and fibrin. The endothelial cells lining the blood vessels are swollen and granular. In places such changes are sufficiently extensive to cause a loss in the continuity of the vessel wall.

Heart, Adrenals, Kidney, Intestines, Bone Marrow, Brain, Ovary, and Uterus.—No significant changes are found in these organs.

---

[1] The findings in the non-pregnant group are in agreement with the observations of Rivers (20) and Stewart (21), and for a more detailed description of the pathology the reader is referred to their papers.

Morbid Anatomy of the Pregnant Rabbits. Liver.—All the rabbits, except one, shown an acidophilic necrosis of the hepatic cells around the central vein (Fig. 3). In some instances the destruction involves practically the entire lobule but there is never any cellular infiltration. In the periportal region of one liver there are a number of stellate elements that resemble the myxomatous cells seen in the spleen. Microscopic examination of the livers from 2 normal rabbits which had just given birth to young reveals that the hepatic cells are filled with fat but are not necrotic.

Skin.—The lesions are not elevated above the surrounding tissue as much as are those in the non-pregnant animals. Histological preparations reveal lesions similar to those observed in the non-pregnant group except that there is less fibrin and the muscle layer appears to be less severely damaged.

Lungs.—All the pregnant animals have secondary lesions in the lungs similar to those described in the non-pregnant animals with the exception that they are much more extensive (Fig. 4). One lung showed a generalized infiltration of the interstitial tissue with large mononuclear cells.

Spleen.—All the spleens are increased in size and the structure is greatly distorted (Fig. 2). Practically no lymphocytes are seen and the Malpighian corpuscles are represented by nests of large stellate cells. Many small foci of necrosis are seen. In the sinuses of the splenic pulp are large amounts of fibrin. The endothelium of the blood vessel is swollen and granular. Macrophages have phagocytized a number of lymphocytes. A few polymorphonuclear cells and some lymphocytes with pyknotic nuclei are seen, but the majority of the cells in the splenic pulp are large stellate elements similar to those seen in the Malpighian corpuscles. Throughout the spleen there is an increase in the number of fibroblasts.

Lymph Nodes.—The amount of endothelial damage and hemorrhage and the number of fibroblasts seem to be greater in the lymph nodes of the pregnant rabbits than in similar structures of the non-pregnant animals.

Ovary and Uterus.—In the muscular layer of the pregnant uteri are seen a few stellate cells resembling the myxomatous cells in the spleen. Around and in the Graafian follicles of the ovaries similar cells are found.

Heart, Adrenals, Kidney, Intestines, Bone Marrow, and Brain.—No significant changes are present in these organs.

From the results of the experiments described above it is obvious that there are differences in the phenomena occurring in pregnant and non-pregnant rabbits infected with the virus of infectious myxomatosis. In pregnant rabbits the lesions at the point of inoculation are less elevated and the secondary lesions of the skin and involvement of the subcutaneous tissues of the ears are either entirely absent or much smaller than are those observed in the control group. More extensive lesions in the spleen and the presence of secondary lesions in the lungs of all the pregnant animals contrast sharply with the involvement of only a few of the Malpighian corpuscles of the spleen and only an occasional secondary lesion in the lungs of the non-pregnant group. A necrosis of the hepatic cells around the central vein occurred in all except one of the pregnant animals, while only a few small areas of central necrosis were seen in 2 of the non-pregnant rabbits. In spite of these differences the animals in the 2 groups lived the same number of days after inoculation.

DISCUSSION

Pregnancy apparently does not change the general resistance of the rabbit to infectious myxomatosis because all the animals, both the pregnant and the non-pregnant, lived the same number of days following inoculation of the virus. The resistance, however, of the various organs in the pregnant animals appears to have been altered—that of the lungs, spleen, and liver is lowered while that of the skin is elevated. This interpretation is of course open to question, as one might maintain that the spleen of the pregnant animal, for example, is more resistant since it evidenced a greater degree of reaction.

In passing one should note that a similar decrease in the resistance of the internal organs of pregnant women to invasion by malignant neoplasms is generally found. The apparent increase in resistance of the skin of pregnant women to certain epitheliomas (15), however, is also of interest in connection with the increased resistance of the skin and subcutaneous tissue of the pregnant animal to infectious myxomatosis.

The conflicting reports of the results of investigation on the influence of pregnancy on the various experimental tumors in animals make a comparison of them with the findings reported here of little value. Furthermore, only the last third of pregnancy was investigated in the present experiments whereas the tumor experiments of other workers extended throughout the whole reproductive cycle, gestation, lactation, and resting phase. It is important to keep this fact in mind, because 2 rabbits inoculated in the earlier period of pregnancy reacted like non-pregnant animals.

The results of this investigation are more comparable to the observations on the effect that acute diseases have on pregnant women, and it is here that we find the most striking similarity, particularly in regard to the reaction of the liver of the pregnant rabbit and the pregnant woman. Acute yellow atrophy occurs more frequently among pregnant than among non-pregnant women. The amount of arsenic that a non-pregnant woman can take with impunity results in an extensive central necrosis of the liver when given to a pregnant woman. The lesion is similar to that seen in the gravid rabbit with myxoma. Such observations indicate that the resistance of the hepatic cells to toxic substances is lowered during pregnancy. A pregnant woman with influenza is much more likely to have pneumonia than is a non-pregnant one (18). In like manner the pregnant rabbit has numerous secondary lesions of the myxoma in the lungs. The decrease in the resistance of the spleen to infectious myxoma resembles the lowered resistance of the spleen of pregnant women to leukemia (22).

SUMMARY

Pregnancy in rabbits alters the reactivity of the tissues to the virus of infectious myxomatosis. The livers of pregnant animals with the myxoma have a central acidophilic necrosis. Secondary lesions in the lungs are much more numerous and larger in the pregnant than in the non-gravid animals. In like manner the lesions in the spleen are more extensive in the pregnant rabbit. On the other hand the skin lesions of the pregnant animal are decreased in size.

REFERENCES

1. Morau, H., *Arch. méd. exp. et anat. path.*, 1894, **6**, 677.
2. Ehrlich, P., *Verhandl. deutsch. path. Ges.*, 1908, **12**, 13.
3. Uhlenhuth and Weidanz, *Arb. k. Gsndhtsamte*, 1909, **30**, 434.
4. Cuénot, L., and Mercier, L., *Compt. rend. Acad.*, 1909, **149**, series 2, 1012.
5. Albrecht, H., and Hecht, V., *Wien. klin. Woch.*, 1909, **22**, 1737.
6. Askanazy, M., *Verhandl. deutsch. path. Ges.*, 1907, **11**, 39.
7. Jentzler, F., *Rev. méd. Suisse romande*, 1908, **28**, 329.
8. Herzog, M., *J. Med. Research*, 1902, **3**, N. S., 74.
9. Kross, I., *J. Cancer Research*, 1921, **6**, 245.
10. Rous, P., *J. Exp. Med.*, 1911, **13**, 248.
11. Fischera, G., *Policlinico, sez. prat.*, Rome, 1909, **16**, 692.
12. Shattock, S. G., Seligmann, C. G., and Dudgeon, L. S., *Proc. Roy. Soc. Med.*, 1910, **3**, path., 127.
13. Slye, M., *J. Cancer Research*, 1920, **5**, 25.
14. Krotkina, N., *Z. Krebsforsch.*, 1924, **21**, 450.

15. Bainbridge, W. S., *Am. J. Obst. and Dis. Women and Child.*, 1918, **77**, 21.
16. Taliaferro, W. H., personal communication.
17. Williams, J. W., Obstetrics, New York, D. Appleton and Co., 5th edition, 1926, 545.
18. Weintraub, S. A., *Mod. Med.*, 1920, **2**, 219.
19. Williams, J. W., Obstetrics, New York, D. Appleton and Co., 5th edition, 1926, 546.
20. Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 965.
21. Stewart, F. W., *Am. J. Cancer*, 1931, **15**, suppl., 2013.
22. Senator, H., and Kaminer, S., Health and disease in relation to marriage and the married state, translated by Dulberg, J., New York, Rebman Co., 1904, 316.

EXPLANATION OF PLATES

PLATE 32

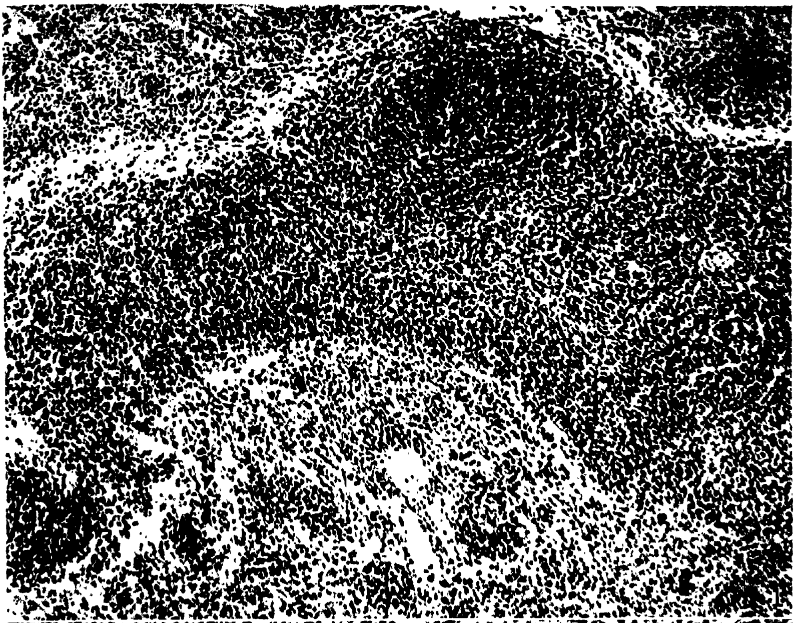
FIG. 1. Histological preparation of a spleen from a non-pregnant rabbit with infectious myxomatosis showing only a slight myxomatous change. Hematoxylin and eosin. × 90.

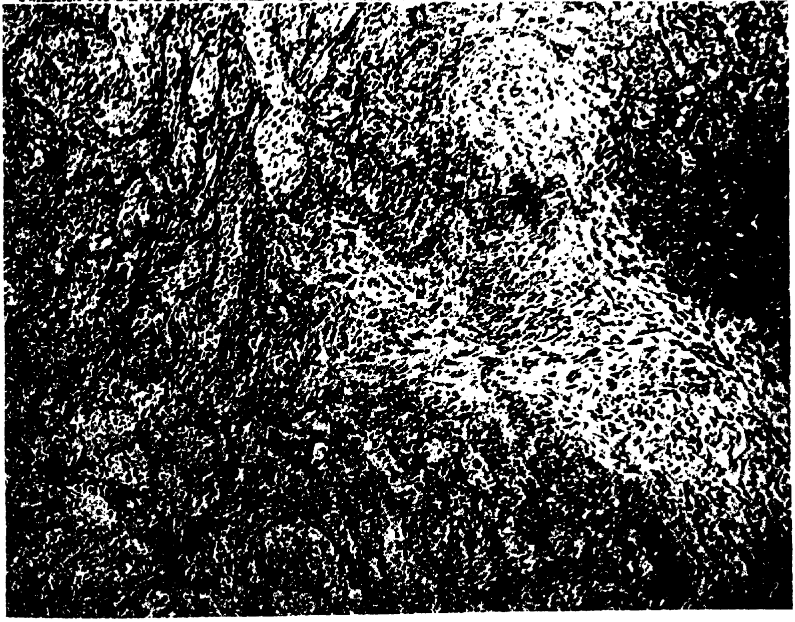
FIG. 2. Histological preparation of a spleen from a pregnant rabbit with infectious myxomatosis showing marked distortion and obliteration of splenic architecture. Hematoxylin and eosin. × 90.

PLATE 33

FIG. 3. Histological preparation of the liver of a pregnant rabbit with infectious myxomatosis showing a central necrosis of the hepatic cells. Hematoxylin and eosin. × 47.

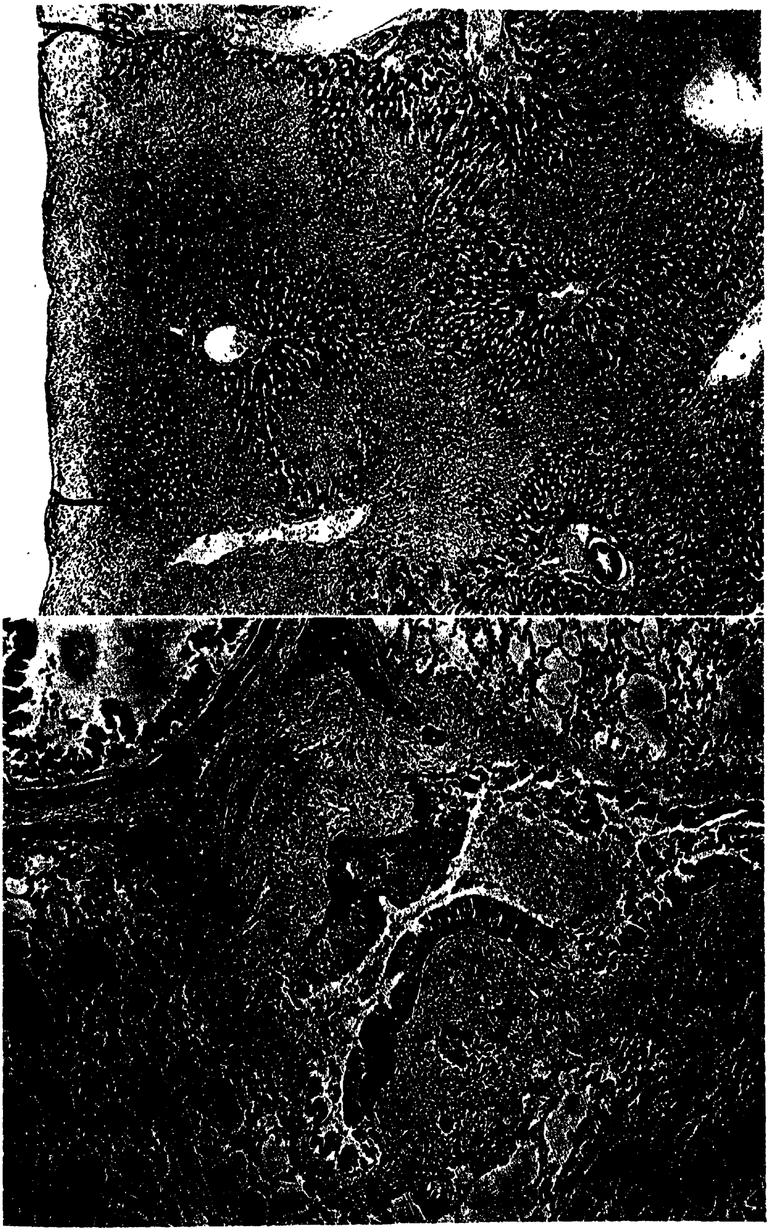
FIG. 4. Histological separation of a lung of a pregnant rabbit with infectious myxomatosis showing secondary lesions around a bronchiole and a hyperplasia of the bronchial epithelium. Hematoxylin and eosin. × 47.





Photographed by Louis Schmidt

(Sprunt: Infectious myxomatosis in pregnant rabbits)



Photographed by Louis Schmidt

(Sprunt: Infectious myxomatosis in pregnant rabbits)

[Reprinted from The Journal of Clinical Investigation, September, 1932, Vol. 11, No. 5, pp. 1037–1052]

THE EFFECT OF NASO-PHARYNGEAL OPERATIONS ON RENAL FUNCTION

By IRVINE H. PAGE and ALF ALVING

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, May 31, 1932)

The operations, tonsillectomy, adenoidectomy, and tooth extraction, are among the most practised therapeutic procedures in Bright's disease. Whether patients suffering from Bright's disease are able, without detriment to their kidneys, to undergo such operations, and whether the operations are likely to affect the subsequent course of the renal disease, are questions which form the object of the present analysis of results. It is based on data from the records of the nephritic clinic of the Hospital of The Rockefeller Institute collected over a period of several years.

Tonsillectomies and adenoidectomies were all performed by Dr. Stuart Craig under gas-oxygen-ether anesthesia, and extractions of teeth by Dr. Frank Wynn using procaine anesthesia. The patients were under the immediate care of Drs. MacKay, Johnston, McIntosh, Moore, Kirk, and Alving.

The cases are classified according to the modification of Volhard and Fahr's classification made by Addis, as described in a previous monograph from this hospital (8).

Data Observed

The "blood urea clearance," indicating the cubic centimeters of blood per minute cleared of urea by renal excretion, has been shown by Van Slyke, McIntosh, Möller, Hannon, and Johnston (7) to be a sensitive indicator of changes in renal function. The urea clearance tests were conducted as proposed by Möller, McIntosh, and Van Slyke (2) with the patient at rest in bed. It is important to stress the latter point because Van Slyke, Alving, and Rose (6) have shown that the clearance in some of the more advanced cases may be significantly reduced by allowing the patient to walk about during the two-hour period of the test. We have recorded the results in percentages of the average normal clearance.

Phenolsulphonphthalein excretion tests were also conducted at the same time as the urea clearance.

Under "duration of disease" the time is given from apparent onset until operation was performed.

Edema, blood pressure, and eye ground examination were usually recorded within a reasonably short period of operation. We have made no effort to call attention to the detail of the eye ground examination, and where we record negative we mean that no gross change, such as hemorrhage and edema, is present.

Hemoglobin is given in terms of oxygen capacity (20.7 volumes per cent being normal for adult men, 19.0 for women) (4).

The normal value for total plasma protein is about 7 per cent. When total protein falls below 5.2 to 5.8 per cent, or the albumin, normally averaging 4.3 per cent, falls below 2.3 to 2.7 per cent, edema is usually present (3).

The Addis sediment test gives the number of red blood cells and casts in a twelve hour specimen collected after a period of twelve hours in which the patient is on a dry diet (1). In many cases it has not been possible immediately after surgical operation to perform this test for obvious reasons. Protein in the urine is recorded for 24 hours, as determined by the sedimentation method of Shevky and Stafford (5).

Histories of Three Cases in Which Renal Function Diminished after Operation

In the series of 31 cases there were 3 in which a marked fall in blood urea clearance followed operation. The histories of these 3 cases are given below. In one of them (No. 1), already in the terminal stage of renal disease and rapidly progressing, the urea clearance fell permanently after operation, which apparently accelerated the fatal course of the disease. In the other two patients the diminution of renal function after operation was temporary, and it does not appear that the progress of the disease was accelerated.

The first patient (No. 1) discovered that he had albuminuria and hematuria during the course of an insurance examination in June 1928. On his admission December 1928, he was edematous, had lost weight, and the blood pressure measured 166/86. Large quantities of albumin, many casts, and red blood cells were found in the urine. The course in the hospital was progressively down hill, terminating in convulsions and death. Features of interest during the course of the disease were, venous thrombosis in the popliteal fossa (December 19), marked increase in the formed elements in the urine following slight upper respiratory infection (March 15), gross hematuria resultant from a streptococcic pharyngitis (April 19), and right bundle branch block on June 9. The urea clearance on admission was 27 per cent of normal and fell progressively to 16 per cent on May 8. On May 11, tonsillectomy and adenoidectomy were performed under gas-oxygen-ether. No gross hematuria was noted after the operation. On May 23, the patient had still not recovered from the operation. He vomited frequently, and was weak, drowsy, and dehydrated. Complete suppression of urine occurred from 6 p.m. on May 23, until he was catheterized at 8 p.m. on May 26. The catheterized specimen contained no acetone or acetoacetic acid, and the specific gravity was 1.017. The plasma pH was 7.31, CO_2 content 20.58 mM. per liter, and chloride 72.1 mM. per liter on May 27. It was not until June 16, that the patient was again bright and cheerful. At that time it was found that the clearance had fallen to 2.4 per cent of normal. Death occurred in uremia on July 2nd.

In this case in spite of difficulty of interpretation due to the rapidly progressive and virulent qualities of the disease it would appear that operation speeded the course.

The second patient (No. 2) suffered from hemorrhagic Bright's disease in the terminal stage. The disease was progressive and caused a rapid loss of kidney function. The patient was continually beset with complications such as abscesses, sties, conjunctivitis, edema, and tonsillitis. His temperature was slightly elevated, his appetite poor, and he had vomited a number of times on previous days. In spite of the adverse condition of the patient, the tonsils were considered so badly infected that their removal was indicated. Following operation his temperature rose to 101.6°, nausea persisted. The ninth day after operation the clearance had fallen to 10 per cent of normal. The patient recovered satisfactorily from the operation but directly following the tonsillectomy the patient seemed to be on the verge of uremia.

The third patient (No. 3) had Bright's disease with a marked nephrotic tendency. On November 17, one tooth was extracted which proved to be normal. There was no fever directly following the operation, although there had been fever for the three previous days. However, for the first time since admission red blood cells in pathological quantities were found in the urine sediment. On November 19, the tooth socket seemed in good condition but the tonsils were swollen and pharyngitis was present. There was a moderate decrease in the urine output, but this decrease had begun three days before the extraction. A marked fall in the urea clearance

TABLE I

Cases in Which Renal Function Diminished after Operation

Case number and clinical data	Date	Blood					Urine (excretion per 12 hours)				Renal function	
				Plasma proteins				Formed elements				
		Urea nitrogen	Hemoglobin	Albumin	Globulin	Total	Protein	Erythrocytes	Leucocytes and epithelial cells	Casts	Phthalein excretion (2 hours)	Urea clearance
	1929	*mgm. per 100 cc.*	*volumes per cent O_2*	*per cent*	*per cent*	*per cent*	*grams*	*millions*	*millions*	*thousands*	*per cent*	*per cent of normal*
No. 1 (6616), male, 17 years. Terminal, hemorrhagic nephritis; previous duration unknown. Blood pressure 120/68, eye grounds negative, edema ++	April 3						5	43.2	60	6900		
	April 10	27										23
	April 16	26	12.4								45	16
	April 24	28		2.1	2.8	4.9	5.5	108.6	171.5	6020	40	17
	May 8	34									27	16
	May 11	Tonsillectomy and adenoidectomy						No especial changes in formed elements after operation				
	June 16	162					5.5	15.7	17.0	460		2.4
	June 24	95										4.2

No. 2 (6238), male, 16 years. Terminal, hemorrhagic nephritis; duration ten weeks. Blood pressure: 148/87 (February 15, 1928), 162/110 (August 16, 1929), 150/110 (December 17, 1929), eye grounds normal, no edema	*1928* February 15			3.0	2.8	5.8						
	March 6	55	12.5					94.0	100.1	6742	14.8	13.2
	March 7	Tonsillectomy—rapid loss of weight and dehydration for one week after operation										
	March 9	48		2.7	2.7	5.5		129.5	192.5	7700	11.8	15.0
	March 16	88	10.6	2.8	3.5	6.4		32.0	43.2	1800	6.7	9.7
	April 2	35		2.4	3.2	5.7		41.0	93.6	1600	10.2	20.4
									Some renal failure casts			
	June 7	48	11.9	3.1	2.3	5.4		60.8	46.4	3040	16.0	17.5
	1929 August 16	54	Plasma specific gravity 1.0249 0.8					3.3	6.8	178	7.5	13.5
	December 17	115	Plasma specific gravity 1.0287 ++++					++++	+++	++	Faint trace	3.2
	December 23	119									0	3.2
	December 28	123										6.4
	December 30	107					++++	++++	+++	++		6.5

TABLE I—*Continued*

Case number and clinical data	Date	Blood					Urine (excretion per 12 hours)				Renal function	
				Plasma proteins				Formed elements				
		Urea nitrogen	Hemoglobin	Albumin	Globulin	Total	Protein	Erythrocytes	Leucocytes and epithelial cells	Casts	Phthalein excretion (2 hours)	Urea clearance
		mgm. per 100 cc.	*volumes per cent O_2*	*per cent*	*per cent*	*per cent*	*grams*	*millions*	*millions*	*thousands*	*per cent*	*per cent of normal*
No. 2 (*concluded*)	*1930* January 6	96										6.5
	January 10	Septal abscess found and incised										
	January 11	84										6.2
	January 22	94					4	24.8	49.6	3650		6.8
	February 3	94									Trace	6.7
	March 3	81										7.6
	March 15	61										11.1

No. 3 (7049), male, 23 years. Initial hemorrhagic nephritis; duration 2 months. Blood pressure 138/86, eye grounds negative, edema ++	*1929* October 19	14	18.1	2.5	3.0	5.6	5.6	0.8		3220	56	61
	October 31	18	19.5	1.9	2.7	4.6	1.0	0.4		944	62	55
	November 17	Tooth extracted (root appeared normal)						Hematuria noted				
	November 21	31	17.8	1.6	2.8	4.5					49	19
	December 5	23		1.6	3.2	4.8	5.8	1.5		6330	72	41
	December 30	22									68	40
	1930 January 27	5	16.8	2.0	2.5	4.6	1.1	1.3		320	81	95
	February 3	20									58	21
	March 10	5									73	94
	August 26	33									18	13
	1931 June 1	19									36	44
	August 4	17		2.5	3.0	5.5	8.0	0.5		4050	58	47

TABLE I—*Concluded*

Case number and clinical data	Date	Blood					Urine (excretion per 12 hours)				Renal function	
		Urea nitrogen	Hemoglobin	Plasma proteins			Protein	Formed elements			Phthalein excretion (2 hours)	Urea clearance
				Albumin	Globulin	Total		Erythrocytes	Leucocytes and epithelial cells	Casts		
		mgm. per 100 cc.	*volumes per cent O_2*	*per cent*	*per cent*	*per cent*	*grams*	*millions*	*millions*	*thousands*	*per cent*	*per cent of normal*
No. 3 (*concluded*)	*1931* August 20	Tonsillectomy and adenoidectomy										
	August 25	18		2.0	3.4	5.5	6.1	0.5		1960	48	50
	September 8	14									46	45
	September 27	15					3.8	0.1		620	54	58

occurred. This would be a rather convincing indication of the effect of the operation on the ability of the kidney to excrete urea had not a very similar episode occurred a month later (January 6 to January 15) when no operative procedure was employed. Oliguria, hematuria and general prostration were evident. After recovery from this incident the clearance rose sharply to 95 per cent on January 27, but fell again to 21 per cent on February 3. From this period his clearance fluctuated markedly and usually without any apparent reason. He seemed peculiarly susceptible to upper respiratory infections. On August 20, of the following year, tonsillectomy and adenoidectomy were performed without effect on the kidney function and the excretion of red blood cells. The patient recovered rapidly from the operation.

DISCUSSION

Except for the three cases which are discussed in detail above, the operations on 31 nephritic patients caused no significant fall or rise in the kidney function as measured either by the urea clearance or phenolsulphonphthalein excretion.

Of the 28 cases who showed no significant renal effects after operation, it has not seemed necessary to report complete data. The observations in Table II covering five of these cases, suffice to show the nature of the results.

The plasma proteins do not appear changed to any evident degree by operation, whereas hemoglobin shows a tendency to fall slightly. Whether the latter is due to the loss of blood at the operation, or is to be credited to the natural progress of the disease is not certain. Urinary protein is not significantly altered. In some cases an increase in hematuria has been noted following operation, in some no change, and in still others a reduction is found. Casts are equally erratic in their appearance. In all respects the effects appear to have been transient, and to have disappeared from a few days to a fortnight after the operation.

The data on the 31 cases discussed above do not cover periods sufficiently long after operation to bear on the effect of the operations on the ultimate course of the disease. However, the charts published in a previous monograph from this hospital (8) do appear to provide some basis for an opinion on this question. Of the 50 cases of acute and chronic hemorrhagic renal disease there reported, seventeen (Numbers 1, 3, 4, 7, 10, 12, 13, 17, 18, 25, 26, 27, 29, 31, 33, 36, 48) were subjected to operation, usually tonsillectomy, and were observed for

TABLE II

Cases without Obvious Change in Renal Function after Operation

Case number and clinical data	Date	Blood					Urine (excretion per 12 hours)				Renal function	
				Plasma proteins				Formed elements				
		Urea nitrogen	Hemoglobin	Albumin	Globulin	Total	Protein	Erythrocytes	Leucocytes and epithelial cells	Casts	Phthalein excretion (2 hours)	Urea clearance
		mgm. per 100 cc.	*volumes per cent* O_2	*per cent*	*per cent*	*per cent*	*grams*	*millions*	*millions*	*thousands*	*per cent*	*per cent of normal*
No. 4 (6538), female, 25 years. Latent, hemorrhagic nephritis for 8 months. Blood pressure 130/90, eye grounds negative, edema ++	*1928* October 23	17	18.2	2.1	3.3	5.4		1.9	3.2	125		55
	November 5	2 teeth roots extracted										
	November 13	9						0.3	1.5	48		90
	December 4	9	18.0	2.2	1.9	4.1		0.03	0.6	18	65	87
	December 15	2 teeth extracted										

No. 4 (*concluded*)	*1929* January 9	11	15.9	2.6	2.5	5.1	2.7	1.1	2.2	259	65	92
	January 24	18	17.1				8.3	0.1	3.1	115	52	96
	February 9	16		2.5	2.2	4.7					59	68
	March 5	8	14.9	2.8	2.7	5.6	1.3	0.3	4.3	60	54	121
	March 14	7	17.1	2.7	2.7	5.5					65	164
	March 27	13	17.9	2.7	2.7	5.5					65	80
	April 6	Molar extracted										
	April 9	9	17.6	2.7	3.2	5.9					60	118
	May 1	9	18.5	3.4	2.3	5.7					60	52
	May 14	12	18.1	3.4	2.5	6.0	1.2	2.8	5.9	71	63	45

TABLE II—*Continued*

Case number and clinical data	Date	Blood					Urine (excretion per 12 hours)				Renal function	
		Urea nitrogen	Hemoglobin	Plasma proteins			Protein	Formed elements			Phthalein excretion (2 hours)	Urea clearance
				Albumin	Globulin	Total		Erythrocytes	Leucocytes and epithelial cells	Casts		
	1926	*mgm. per 100 cc.*	*volumes per cent* O_2	*per cent*	*per cent*	*per cent*	*grams*	*millions*	*millions*	*thousands*	*per cent*	*per cent of normal*
No. 5 (5681), male, 8 years. Active, chronic hemorrhagic nephritis for 4 months. Blood pressure 126/70, eye grounds negative, edema ++	May 3	36									53	30
	May 6	36	14.2	2.1	2.1	4.2	3.6	++++	++++	+++		20
	May 17	25					4.0	++++	+++	+++	15	11
	May 21	Tonsillectomy										
	May 28	29					5.0	++++	++	++	10	20
	June 3	30	8.2				6.0	++++	++	++	8	20
	June 22	26	9.1	2.4	2.4	4.8	4.0	Gross			8	20
	July 8	28					2.8	++++	++	++	10	19
	July 16	29					4.0	+++	+++	++	8	30
	July 29	20					3.0	++++	++	++	29	32
	August 16	19	11.0				2.5	++++	++	+		40
	August 27	17	13.2	2.7	2.4	5.2	4.2	++++	++	+	38	43
	September 10	19	10.8				3.0	++++	++	+	62	41
	September 20	14	13.8				2.1	++++	++	+	62	70

No. 6 (7090), female, 15 years. Active, chronic hemorrhagic nephritis for 14 months. Blood pressure 140/80, eye grounds negative, no edema	*1929* April 21	25									52	32
	May 3	16					3.6	40.4	22	3800	63	39
	June 7						3.2	14.3	60	2800		
	June 9	12									52	61
	June 18	15					2.4	18.8	14	2800		44
	June 19	Tonsillectomy										
	June 23	17									45	43
	July 2	16									38	27
	July 3	17										34
	July 31	17	17.5								45	43
	September 29	21					17.5	15.8	76	2200	41	57
	October 1	20							*			45

TABLE II—*Concluded*

Case number and clinical data	Date	Blood					Urine (excretion per 12 hours)				Renal function	
		Urea nitrogen	Hemoglobin	Plasma proteins			Protein	Formed elements			Phthalein excretion (2 hours)	Urea clearance
				Albumin	Globulin	Total		Erythrocytes	Leucocytes and epithelial cells	Casts		
		mgm. per 100 cc.	*volumes per cent O_2*	*per cent*	*per cent*	*per cent*	*grams*	*millions*	*millions*	*thousands*	*per cent*	*per cent of normal*
No. 7 (7094), female, 26 years. Active, chronic hemorrhagic nephritis for 21 months. Blood pressure 226/168, eye grounds—retinal arteries narrowed, edema ++	*1929* December 16	49					1.8	4.6	7.5	905		34
	December 18	48										36
	1930 January 8	18	16.9	1.7	2.7	4.4						31
	January 27	30										38
	January 29	Tonsillectomy										
	February 2	30		2.8	2.1	4.9						34
	February 11	28										41
	February 17	29	15.1				2.2	0.08	20.6	1504		38
	March 17	32					1.0	0.08	41.3	1474		39
	March 24	23										39

No. 8 (7388), male, 24 years. Active and latent hemorrhagic nephritis for 15 months. Blood pressure 152/88, eye grounds, small old white plaque near disc, edema +	*1930* April 21	12	18.3	3.0	2.2	5.2	0.7	3.1	4.2	19	67	65
	May 3	17					1.1	1.4	3.4	58	66	57
	May 3	Abscessed tooth removed										
	May 5	16									72	55
	May 19	16									71	33
	June 9	7					1.2	2.0	1.6	115	71	80
	June 18	6									70	78
	June 19	Tonsillectomy and adenoidectomy										
	June 28	20						No reaction in urine			61	25
	June 30	9									79	84
	July 29						1.2	0.6	1.7	136		
	September 30	12					2.0	0.3	0.6	32	50	77
								No hematuria has been found since on two yearly visits				

months or years thereafter. Comparison of these charts with those of similar cases that were not subject to operation gives, we believe, a definite impression that the tonsillectomies were without effect, either favorable or unfavorable, on the course of the disease. The natural variability of the disease makes it impossible to state dogmatically this conclusion. We believe, however, that anyone who will compare the series of charts referred to with charts of unoperated cases in the same monograph will have difficulty in finding evidence for any effect of the operation on the course of the renal disease.

Experience with these and other cases has led to the conclusion in this clinic that in nephritic patients removal of tonsils, adenoids, and teeth are operations to be undertaken or not from the standpoint of the general hygiene of the patient, and without expectation that his renal disease will be benefited. On the other hand, unless the general condition is bad, it does not seem necessary to refuse an operation because of a somewhat diminished renal function. If the disease has been very active and the general condition poor, there may be danger of bringing on uremia in the days following the operation. But such danger seems to be remote, in subjects with blood urea clearances above 20 per cent of normal, if the general condition is not depressed by factors other than renal retention.

CONCLUSIONS

The operations of tonsillectomy, adenoidectomy, and tooth extraction have usually had no effect on kidney function as measured by the urea clearance or the phthalein excretion. In 3 out of 31 cases, however, renal function was depressed in the days immediately after operation, and in one case, already terminal, uremic symptoms were brought on.

2. Red blood cells and casts in the urine were frequently increased for some days after operation. Such increase usually occurred without any fall in renal function.

3. Plasma protein content and urinary protein excretion were not affected by these operations. The hemoglobin content of the blood was in some cases somewhat decreased, perhaps as the result of loss of blood during the operation.

4. The results, together with more prolonged observations pre-

sented in a previous report (8), lead to the conclusions that removal of tonsils, adenoids, or teeth usually has no effect, favorable or unfavorable, on the progress of renal disease, but that when the general hygiene of a renal patient demands such an operation it is not necessary to refuse it, unless the renal disease is in the terminal stage (urea clearance consistently below 20 per cent of normal), or the general condition of the patient is unfavorable.

BIBLIOGRAPHY

1. Addis, T., J. Am. Med. Assoc., 1925, lxxxv, 163. A clinical classification of Bright's disease.
2. Möller, E., McIntosh, J. F., and Van Slyke, D. D., J. Clin. Invest., 1928, vi, 485. Studies of urea excretion. IV. Relationship between urine volume and rate of urea excretion by patients with Bright's disease.
3. Moore, N. S., and Van Slyke, D. D., J. Clin. Invest., 1930, viii, 337. The relationships between plasma specific gravity, plasma protein content and edema in nephritis.
4. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Volume I. Interpretations, The Williams & Wilkins Co., Baltimore, 1931, p. 544.
5. Shevky, M. C., and Stafford, D. D., Arch. Int. Med., 1923, xxxii, 222. A clinical method for the estimation of protein in urine and other body fluids.
6. Van Slyke, D. D., Alving, A., and Rose, W. R., (unpublished observations).
7. Van Slyke, D. D., McIntosh, J. F., Möller, E., Hannon, R. R., and Johnston, C., J. Clin. Invest., 1930, viii, 357. Studies of urea excretion. VI. Comparison of the blood urea clearance with certain other measures of renal function.
8. Van Slyke, D. D., Stillman, E., Möller, E., Ehrich, E., McIntosh, J. F., Leiter, L., MacKay, E. M., Hannon, R. R., Moore, N. S., and Johnston, C., Medicine, 1930, ix, 257. Observations on the courses of different types of Bright's disease and on the resultant changes in renal anatomy.

[Reprinted from THE JOURNAL OF CLINICAL INVESTIGATION, September, 1932, Vol. 11, No. 5, pp.1053–1064]

STUDIES OF UREA EXCRETION

VII. THE EFFECTS OF POSTURE AND EXERCISE ON UREA EXCRETION

BY D. D. VAN SLYKE, A. ALVING, AND W. B. ROSE

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, May 31, 1932)

The studies reported in this paper were undertaken primarily to ascertain whether, in determining the blood urea clearance[1] as a measure of renal function, the previously utilized precaution of keeping the subject at rest in a recumbent position is necessary, and whether the mild exercise and change of posture associated with walking about has sufficient effect on renal activity to alter the clearance values obtained. Observations in the two conditions have been made on three normal men and nineteen nephritic subjects with varying grades of renal impairment. The effect of change of posture from lying in bed to sitting in a chair has also been studied. Furthermore, in three subjects with normal renal function, the effect was studied of exercise of the maximum severity which could be maintained for the 2-hour period.

There is evidence that renal function in many details is affected by mere change in posture. Edel (5) in 1901 observed that change from the recumbent to the standing position decreased markedly the volume output of urine, and in subjects with cyclic albuminuria increased the protein excretion. Subsequent authors (6, 9, 15, 17, 22) have confirmed this observation, and have found that the excretion rate, not only of water, but of most of the other normal urinary constituents, is diminished by the change from recumbent to standing posture. Thus White, Rosen, Fischer, and Wood (22) found that the average effect in three normal men of changing from lying to standing posture was to diminish the hourly excretions to the following proportions of the outputs in the recumbent position: urine volume to 30 per cent, chlorides to 45 per cent, phosphorus to 67 per cent, sulfur to 71 per cent, urea to 61 per cent, and creatinine to 91 per cent. Cordero and Friedman (4) found that change from recumbent to upright position caused an average decrease of 10 to 13 per cent in the proportion of injected phenolsulfonephthalein excreted in 2 hours by normal subjects.

---

[1] The terms, "standard blood urea clearance" and "maximum blood urea clearance," used in expressing the urea excreting efficiency of the kidneys, have been defined in the second paper of this series (12).

As possible causes of such effects, Erlanger and Hooker (6) and White, Rosen, Fischer, and Wood (22) observed that the change from recumbent to upright position caused in 30 to 60 minutes a concentration of the blood by seepage of 10 to 11 per cent of the total plasma fluid into the leg tissues, and also that there was a decrease of 10 to 15 millimeters in pulse pressure. That the urine flow is diminished because increased osmotic pressure of the more concentrated plasma proteins retards glomerular filtration and favors tubular reabsorption of water, was believed by Ni and Rehberg (15), while Erlanger and Hooker (6) were inclined to see the chief retarding factor in the decreased pulse pressure and the number of functioning glomeruli.

Whether the urea clearance is affected depends upon whether the relationship between the outputs of urea and water is that allowed for in calculating the clearance. When the volume is below the augmentation limit of about 2 cc. per minute, the calculation of the clearance allows for change of urea excretion rate in proportion to the square root of the urine volume, while with higher volumes urea excretion rate, estimated as the "maximum clearance" (11), is assumed to be independent of urine volume. If standing up does not retard the urea output more than accords with the above allowances for urine volume effect, the value of the clearance will not be affected.

In regard to this question the only data available appear to be observations made by Addis and Drury (1) and MacKay (10) on the effects of vigorous exercise. Addis and Drury found that in a normal subject the maximum blood urea clearance during an hour of continuous running was decreased to 70 per cent of the value observed during rest. MacKay found that during a 2-hour tennis match the standard clearance of a normal man was lowered to about half its usual resting value.

In previous studies by Möller, McIntosh, and Van Slyke (12, 13) administration of 15 grams of urea before the observation period was found to have no significant influence on the clearances of either normal or nephritic subjects resting in bed. Professor G. A. Harrison of St. Bartholomew's Hospital in London has personally communicated to the writers some observations of blood urea clearances during successive hourly periods in several subjects who were up and about. In some cases 15 grams of urea were given before the day's series of observations, while on other days the same subjects were observed without urea administration. It appeared from Professor Harrison's preliminary observations that urea administration to subjects who were up and about might exert a stabilizing effect on their clearance. Professor Harrison did not wish to pursue the question farther. We have accordingly included in the present studies observations in which urea was given and withheld on alternate experimental days.

Methods

The *standard blood urea clearance*, $C_s = \frac{U}{B}\sqrt{V_c}$, (11, 12) was calculated in all instances in which the volume of urine, corrected for surface area (11) was below the augmentation limit of 2 cc. per minute. When the corrected volume was above this limit the *maximum blood urea clearance*, $\left(C_m = \frac{UV_c}{B}\right)$, was used. In the above formulae, U represents the urine urea concentration, B the blood urea concentration, and V_c the urine volume in cubic centimeters per minute, corrected for body size as described by McIntosh, Möller, and Van Slyke (11). On the charts and in the tables the clearance has been recorded in percentages of the normal mean standard clearance of 54 cc. per minute or of the normal mean maximum clearance of 75 cc. per minute.

The blood and urine urea concentrations were determined by the gasometric urease method (19).

All blood urea clearance tests were performed in the morning. Each patient received at 7 a.m. an ordinary breakfast, unrestricted except for coffee, which was uniformly omitted. Urine was voided at 8:30 a.m. and the specimen was discarded. The urines voided during the two 1-hour periods 8:30–9:30 and 9:30–10:30 were then collected separately. At about 9:30 blood was drawn for urea determination.

Whenever feasible, observations were carried out while the subjects were, on alternate days, in bed and walking about, or in bed and sitting up. Similar studies were made after urea ingestion, 15 grams of urea by mouth being given on the mornings of these tests.

One patient (No. 19), normal except for a slight albuminuria, exercised vigorously by walking up and down the stairs of the hospital on the day of the test. Two normal individuals exercised by playing squash-racquets during the 2 hours of the tests.

Clinical details other than the blood urea clearance will not be given, because they do not appear of significance to these conclusions. The subjects represented in Figure 1 were separable with regard to renal condition as follows: Nos. 1, 2, 3, 4, 5, 6, 7, and 10, were in the terminal stage of hemorrhagic Bright's disease, as defined in a previous paper (21). Nos. 9, 12, 16, 17, and 18 were in the active chronic stage of the same disease. Nos. 11 and 20 were degenerative, and 13 was sclerotic. Nos. 19, 21, 22, and 23 were normal young adults, except that 19 had an occasional slight albuminuria.

RESULTS

The urea clearance values are shown in Figure 1. In Tables 1 and 2 the data in detail are given for the observations on one normal subject and on one nephritic, Subject 6. The latter case was selected for presentation in detail because the subject, more than any other in our series, shows the depressing effect on urea excretion as the result of changing from bed rest to walking about.

The Effect of Mild Physical Activity on the Blood Urea Clearance

In normal subjects, and in nephritic patients with relatively slight functional impairment (over 50 per cent of normal clearance) (Subjects 13 to 23), being up and about caused no significant change in the urea clearance from the values observed when the subjects were in bed before and during the excretory periods. In some cases the average clearance obtained when the subjects were up and about was lower,

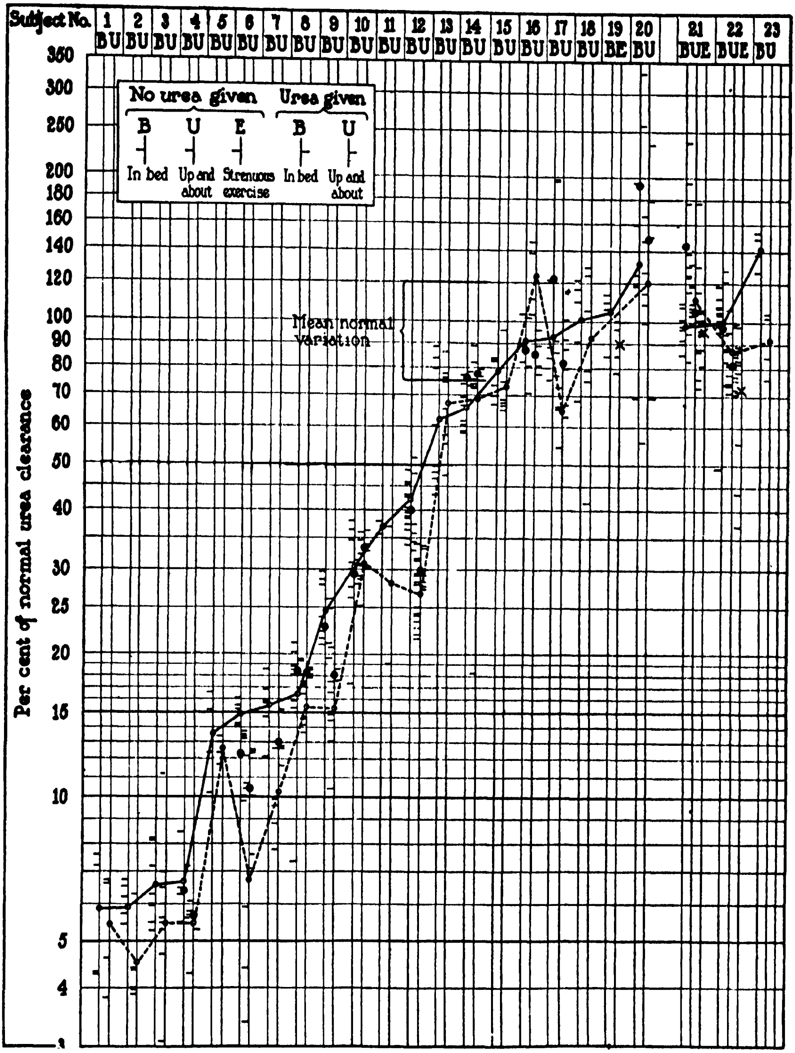


FIG. 1

Solid line connects average clearance values taken while subjects were in bed. Dashed line connects average clearance values taken while subjects were up and about. Hollow circle indicates average of all clearances on a subject, solid circle indicates average of clearances taken after administration of urea. × mark indicates average of clearances taken during hard exercise. Short horizontal lines indicate individual clearance values.

TABLE 1

Observations on A. H., Terminal Hemorrhagic Nephritic, Showing Maximal Effect on Urea Excretion Caused by Change from Bed Rest to Moderate Activity

Case No. 6: Hospital No. 7525

Conditions of observation		Date	V_{cor}	U	B	$\frac{U}{B}$	Blood urea clearance $\frac{U\sqrt{V}}{B}$ or $\frac{UV}{B}$
		1930–31	*cc. urine per minute*	*mgms. urea N per 100 cc. urine*	*mgms. urea N per 100 cc. blood*	*Concentration ratio*	*per cent of normal*
No urea given	In bed	November 25	0.560	436	42.6	10.2	14.2
		"	1.12	350	42.6	8.2	16.1
		November 28	0.608	431	44.2	9.8	14.1
		"	0.832	407	44.2	9.2	15.5
		December 9	0.592	404	41.0	9.9	14.1
		"	0.896	357	41.0	8.7	15.3
		Average	0.77	398	42.6	9.3	14.9
	Up and about	November 26	0.144	202	42.1	4.8	3.4
		"	0.464	405	42.1	9.6	12.2
		November 29	0.256	274	43.5	6.3	5.9
		"	0.400	387	43.5	8.9	10.4
		December 10	0.126	272	41.0	6.6	4.4
		"	0.320	417	41.0	10.2	10.7
		Average	0.29	326	42.2	7.7	7.8
15 grams urea by mouth before each test	In bed	December 16	1.47	332	55.1	6.0	13.5
		"	1.12	359	55.1	6.5	12.8
		December 18	1.50	444	72.8	6.1	13.8
		"	1.95	381	72.8	5.2	13.5
		January 13	0.895	471	82.6	5.7	10.0
		"	0.943	451	82.6	5.5	9.8
		Average	1.31	406	70.2	5.8	12.2
	Up and about	December 17	0.416	534	63.2	5.5	10.1
		"	0.704	507	63.2	6.7	12.5
		December 19	0.880	526	72.2	6.8	12.6
		"	0.768	556	72.2	6.8	12.5
		January 14	0.480	545	92.1	4.1	7.6
		"	0.416	546	92.1	3.8	7.1
		Average	0.611	536	75.8	7.2	10.4

TABLE 2

Observations on A. A., Normal Subject, No. 22

Conditions of observation		Date	V_{cor}	U	B	$\frac{U}{B}$	Blood urea clearance $\frac{U\sqrt{V}}{B}$ or $\frac{UV}{B}$
		1931	*cc. urine per minute*	*mgms. urea N per 100 cc. urine*	*mgms. urea N per 100 cc. blood*	*Concentration ratio*	*per cent of normal*
No urea given	In bed	January 6	2.61	533	15.6		119
		"	4.09	306	15.6		107
		January 13	1.23	871	18.3	48	98
		"	2.30	289	18.3		(48)*
		January 23	1.22	879	16.7	52	107
		"	1.53	722	16.7	43	99
		February 10	1.52	572	13.7	42	95
		"	2.72	425	13.7		112
		February 12	1.58	747	16.7	45	104
		"	1.85	687	16.7	41	104
		Average	2.07	603	16.2	45	105
	Up and about	January 7	0.979	752	15.8	48	87
		"	1.03	681	15.8	43	81
		January 14	0.710	1148	18.9	61	95
		"	0.896	1148	18.9	61	106
		January 22	1.23	837	22.3	38	77
		"	1.20	775	22.3	35	71
		January 30	0.957	853	15.7	54	106
		"	0.833	761	15.7	49	100
		February 3	0.458	964	16.5	59	74
		"	0.537	896	16.5	55	74
		Average	0.880	882	17.8	50	84
	Vigorous exercise	January 8	0.576	950	16.3	58	82
		"	0.945	878	16.3	54	97
		January 15	0.803	920	19.6	47	78
		"	0.831	957	19.6	49	83
		January 31	0.437	1101	15.5	71	87
		"	0.453	1098	15.5	71	88
		March 28	0.265	1044	18.1	58	56
		"	0.365	1155	18.1	64	71
		March 29	0.349	557	16.4	34	37
		"	0.409	603	16.4	37	44
		Average	0.544	926	17.2	54	72

* Omitted from average.

TABLE 2—*Concluded*

Conditions of observation		Date	V_{cor}	U	B	$\frac{U}{B}$	Blood urea clearance $\frac{U\sqrt{V}}{B}$ or $\frac{UV}{B}$
		1931	*cc. urine per minute*	*mgms. urea N per 100 cc. urine*	*mgms. urea N per 100 cc. blood*	*Concentration ratio*	*per cent of normal*
15 grams urea by mouth before each test	In bed	January 20	1.76	956	26.9	36	87
		"	2.14	821	26.9		87
		February 5	2.63	946	27.2		122
		"	2.95	885	27.2		128
		February 13	1.41	1165	34.7	40	74
		"	1.55	1236	34.7	44	82
		February 14	1.39	1164	30.3	54	101
		"	1.85	1153	30.3	52	96
		Average	2.04	1041	29.8	45	97
	Up and about	January 9	1.21	1259	30.4	41	84
		"	0.871	1359	30.4	44	77
		January 10	1.50	1444	33.7	43	97
		"	1.23	1418	33.7	42	87
		January 21	1.78	904	29.7	30	75
		"	1.19	1036	29.7	35	71
		February 4	2.03	944	28.0		91
		"	1.87	798	28.0	28	72
		Average	1.46	1145	30.5	37	82

in others higher, than when in bed. As shown by the data of Table 2, given in full for a normal subject, the most marked renal effect of the change in posture and activity is in the urine volume, much less being excreted while the subject is up and about than when in bed. This phenomenon was uniformly noted both in the subjects with normal renal function and in those with diminished function. The urea concentration in the smaller volume of urine was increased sufficiently, however, to prevent the clearance figures from varying, except in one or two isolated observations, markedly outside the range of clearances observed during bed rest in this group of subjects (Nos. 13 to 23).

In nephritic subjects with less than 50 per cent of normal clearance (Subjects 1 to 12 inclusive) being up and about uniformly depressed the

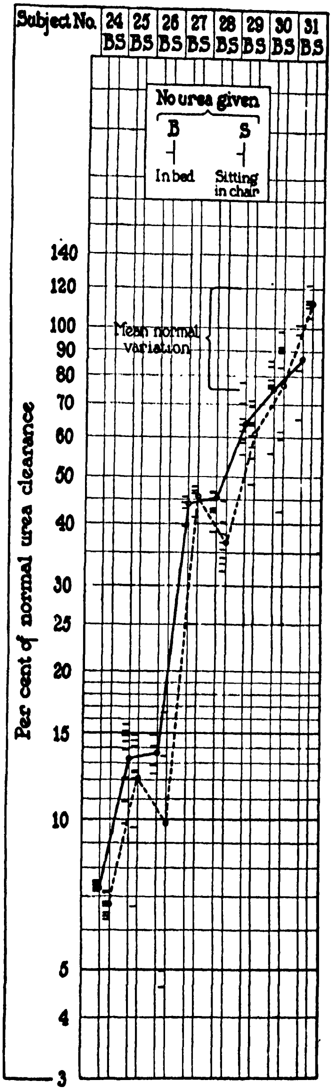


FIG. 2

Solid line connects average clearance values taken while subjects were in bed. Dashed line connects average clearance values taken while subjects were sitting. Short horizontal lines indicate individual clearance values.

average clearance below that observed during bed rest, except in one patient (No. 10) where there was no change. In three-fourths of the cases the effect was relatively slight and not sufficient to influence the diagnostic import of the clearances, but in cases 6, 9, and 12 being up and about lowered the mean clearance to 60 per cent or less of that observed during bed rest. It is evident therefore that in nephritics with marked renal impairment clearance determinations should continue to be made under standard conditions of rest.

During approximately half of another series of clearance tests each subject sat in a chair. The other clearances of the series were taken with the subjects in bed. The results are shown in Figure 2. They indicate no demonstrable effect of this change of posture on the clearance.

The Effect of Severe Exertion on Urea Excretion in Subjects with Normal Renal Function

Severe exertion was tested in three subjects, prolonged stair climbing in Subject 19, and squash-racquets playing in Nos. 21 and 22. The results with Subject 22 are given in detail in Table 2. The severe exercise diminished the average clearance somewhat more than did the mild exercise of merely being up and walking about. The effect of the severe exercise on urea excretion was not great, however, except in two clearances in Subject 22, which were performed after he had exercised nearly to exhaustion, and were found reduced to 35 and 44 per cent of normal. Nearly all of the clearances taken during severe exercise lay within the range normally encountered in subjects resting in bed, the slightly depressing effect of the exercise being apparent only statistically. Presumably severe exercise would depress more markedly the renal function in advanced nephritis.

The Effect of Urea Administration on the Renal Reaction to Physical Activity

The data in Figure 1 reveal no effect of urea administration on the clearances, either when the subjects were resting in bed or were up and about. In this respect the results confirm those of previous studies by Möller, McIntosh, and Van Slyke (12, 13).

The Effect of Exercise on Urine Volume

As illustrated by the data in Tables 1 and 2, the urine volume in both normal and nephritic subjects was observed to be markedly lower while a subject was up and about than when he was in bed. The relative oliguria was probably due to the change to upright posture, which is known to diminish urine volume (6, 9, 15, 17, 22), rather than to the mild muscular exertion.

In the very severe exercise taken by Subjects 19, 21, and 22, diminution of urine volume is still more marked. It is probable that some dehydration of the blood by sweating occurred, and perhaps also some diversion of blood flow from the kidneys to the heavily working muscles.

Variations of Urine Concentration

In a normal subject, blood urea being constant, the urea concentration in the urine is greater when the volume of urine excreted per minute is less. When, with constant blood urea content, the urine volume is less than the "augmentation limit" of about 2 cc. per minute, the urea concentration in the urine ordinarily varies inversely as the square root of the volume: if the volume is reduced to $\frac{1}{4}$ the concentration is multiplied by 2 (3, 12). Such a relationship is approximated by the data in Table 3 from a normal subject, and is not markedly disturbed by exercise, even when severe. (The three sets of data taken with "no urea given" can be compared together, while the two sets taken after urea administration form another group comparable among themselves, because of approximate constancy of the blood urea content.) Möller, McIntosh, and Van Slyke (12) found a similar behavior in nephritics of all grades of severity when the subjects were tested during rest in bed. The concentrations of urea in the urine compared with the concentrations in the blood were much smaller in advanced nephritis than in normal subjects, but the variation with urine volume in a given subject was of the same nature.

In nephritic subjects Nos. 6, 9, 12, however, who show marked fall in blood urea clearance caused by leaving bed and walking about, this inverse relationship between urine volume and concentration fails to hold. In No. 6 (Table 1), who shows, of the subjects reported in this paper, the most depression of clearance caused by leaving bed and walking about, there is in the first two sets of data, taken without urea administration, no inverse relationship at all. The urine volume, in the observations made when this subject was up and about averages only 40 per cent as great as the volume when he was in bed, but the urea concentration in the smaller volume, when the patient was up, averages actually somewhat less than in the larger volumes excreted in bed. It is this failure, partial or complete, to increase normally the urea concentration in the smaller volumes of urine passed while up and about, that causes the clearance to be markedly depressed by the latter condition in three of the nineteen nephritics studied. The depression, although evident only in nephritic cases with less than 50 per cent of normal clearance, was not at all directly related to the severity of renal impairment. In some of the most advanced cases being up and about depressed the clearance but little.

SUMMARY

In normal subjects, or in nephritics with more than 50 per cent of normal renal function measured by the blood urea clearance, the clearance has not been found to be essentially different, whether the subjects were kept in bed during the 2-hour observation periods or were walking about. Urine volume was uniformly less while the subjects were up and about, but the effect in depressing urea excretion was about that allowed for in the formulae for calculating the standard and maximum clearances, so that the values of the latter were not markedly affected.

In three of the twelve nephritics observed with less than 50 per cent of normal renal function, measured by the blood urea clearance observed with the subjects in bed, rising and walking about depressed the clearance markedly, the mean values observed being 44, 60, and 67 per cent respectively of those observed in the same patients during bed rest.

Severe exercise taken by three subjects with normal renal function depressed the clearance somewhat, as found by Addis and Drury (1) and MacKay (10), but in only three out of twenty-two clearances determined during heavy exertion were the values definitely abnormal, i.e, below 70 per cent of the mean normal level.

Administration of 15 grams of urea before the observation periods did not significantly affect the results.

It appears that in normal subjects and in nephritics with more than 50 per cent of average normal clearance, the clearance values can be determined without loss of accuracy while the subjects are up and about. In nephritic patients with less than 50 per cent of normal clearance values, however, it is essential to keep the subject at rest in a recumbent position during the 2-hour excretion period, if the results are to be compared with those heretofore obtained by the clearance test.

BIBLIOGRAPHY

1. Addis, T., and Drury, D. R., J. Biol. Chem., 1923, lv, 629. The rate of urea excretion. VII. The effect of various other factors than blood urea concentration on the rate of urea excretion.
2. Addis, T., J. Am. Med. Assoc., 1925, lxxxv, 163. A clinical classification of Bright's disease.
3. Austin, J. H., Stillman, E., and Van Slyke, D. D., J. Biol. Chem., 1921, xlvi, 91. Factors governing the excretion rate of urea.
4. Cordero, N., and Friedman, M. H., Arch. Int. Med., 1928, xli, 279. Influence of posture on phenolsulphonephthalein test for kidney function.
5. Edel, P., Munch. med. Wchnschr., 1901, xlviii, 1833. "Cyklische" Albuminurie und neue Gesichtspunkte für die Bekämpfung von Albuminurien.
6. Erlanger, J., and Hooker, D. R., Johns Hopkins Hosp. Rep., 1904, xii, 357. An experimental study of blood pressure and of pulse pressure in man.
7. Howe, P. E., J. Biol. Chem., 1921, xlix, 109. The determination of proteins in blood—a micro method.
8. Lashmet, F. H., and Newburgh, L. H., J. Am. Med. Assoc., 1930, xciv, 1883. The specific gravity of the urine as a test of kidney function.
9. Linossier, G., and Lemoine, G. H., Compt. rend. Soc. de biol., 1903, lv, 466. Influence de l'orthostatisme sur le fonctionnement du rein.
10. MacKay, E. M., J. Clin. Invest., 1928, vi, 505. Studies of urea excretion. V. The diurnal variation of urea excretion in normal individuals and patients with Bright's disease.
11. McIntosh, J. F., Möller, E., and Van Slyke, D. D., J. Clin. Invest., 1928, vi, 467. Studies of urea excretion. III. The influence of body size on urea output.
12. Möller, E., McIntosh, J. F., and Van Slyke, D. D., J. Clin. Invest., 1928, vi, 427. Studies of urea excretion. II. Relationship between urine volume and the rate of urea excretion by normal adults.
13. Möller, E., McIntosh, J. F., and Van Slyke, D. D., J. Clin. Invest., 1928, vi, 485. Studies of urea excretion. IV. Relationship between urine volume and rate of urea excretion by patients with Bright's disease.

14. Moore, N. S., and Van Slyke, D. D., J. Clin. Invest., 1930, viii, 337. The relationships between plasma specific gravity, plasma protein content and edema in nephritis.
15. Ni, T., and Rehberg, P. B., J. Physiol., 1931, lxxi, 331. On the influence of posture on kidney function.
16. Rowntree, L. G., and Geraghty, J. T., J. Pharm. and Exp. Therap., 1910, i, 579. An experimental and clinical study of the functional activity of the kidneys by means of phenolsulphonephthalein.
17. Thompson, W. O., Thompson, P. K., and Dailey, M. E., Proc. Nat. Acad. Sci., 1928, xiv, 94. The effect of posture upon the composition and volume of blood in man.
18. Van Slyke, D. D., J. Biol. Chem., 1927, lxxi, 235. Gasometric micro-Kjeldahl determination of nitrogen.
19. Van Slyke, D. D., J. Biol. Chem., 1927, lxxiii, 695. Determination of urea by gasometric measurement of the carbon dioxide formed by the action of urease.
20. Van Slyke, D. D., and Hiller, A., J. Biol. Chem., 1928, lxxviii, 807. Gasometric determination of hemoglobin by the carbon monoxide capacity method.
21. Van Slyke, D. D., Stillman, E., Möller, E., Ehrich, E., McIntosh, J. F., Leiter, L., MacKay, E. M., Hannon, R. R., Moore, N. S., and Johnston, C., Medicine, 1930, ix, 257. Observations on the courses of different types of Bright's disease and on the resultant changes in renal anatomy.
22. White, H. L., Rosen, I. T., Fischer, S. S., and Wood, G. H., Am. J. Physiol., 1926, lxxviii, 185. The influence of posture on renal activity.

[Reprinted from THE JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION, October 15, 1932, Vol. 99, No. 16, p. 1344]

A SIMPLE TEST FOR PLASMA PROTEIN CONTENTS BELOW THE EDEMA-PRODUCING LEVEL

BY IRVINE H. PAGE, M.D., AND DONALD D. VAN SLYKE, PH.D., SC.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

A type of edema called "hydremic" has long been recognized in which the blood plasma is abnormally dilute with regard to its protein content. Such edema is encountered in nephritis, diabetes and other conditions in which malnutrition leads to plasma protein deficit. That this type of edema is due to diminished plasma protein content and to the consequent diminution of the osmotic attraction of the blood for fluid from the tissue spaces is a conclusion based on numerous physiologic experiments and clinical observations.[1] Furthermore, in a study of the quantitative relationship between plasma protein deficit and the tendency to edema in nephritic patients, Moore and Van Slyke[2] found that a critical level of the plasma protein concentration exists above which there is usually no tendency toward the formation of edema and below which such tendency is regularly present. This critical level is about 5.3 per cent of total plasma protein and is accompanied by a specific gravity of approximately 1.023. Liu[3] found the same critical level in edema attributable to plasma protein deficit caused by malnutrition. In nephritis, diabetes and other conditions involving malnutrition, data showing whether the plasma protein content or specific gravity is above or below this critical level assist in deciding whether the tendency to edema and ascites of the "hydremic" type, from protein deficit, is present, and in differentiating this type of edema or ascites from types due to other causes.

---

[1] This evidence, first applied clinically by Epstein, has been reviewed recently by Peters and Van Slyke: Quantitative Clinical Chemistry **1**: 682.

[2] Moore, N. S., and Van Slyke, D. D.: The Relationships Between Plasma Specific Gravity, Plasma Protein Content and Edema in Nephritis, J. Clin. Investigation **8**: 337 (April) 1930.

[3] Liu, S. H.; Chu, H. I.; Wang, S. H., and Chung, H. L.: Nutritional Edema: I. Effect of Level and Quality of Protein Intake on Nitrogen Balance, Plasma Proteins, and Edema, Proc. Soc. Exper. Biol. & Med. **29**: 250 (Dec.) 1931.

Of such other types, the edema of cardiac failure is probably the one most frequently requiring differentiation from "hydremic" edema.

For a simple test to determine whether the plasma specific gravity is above or below the critical level we have utilized the familiar principle of observing whether a drop of the plasma sinks or floats in another liquid of known specific gravity. We have obtained the desired specific gravity, viz., 1.0235 at 20 C. (water at 20 C. being taken as 1.000) by using monofluorobenzene,[4] or a mixture of one volume of xylene with 2.06 volumes of monochlorobenzene. The fluorobenzene has the advantage that the specific gravity of the pure substance at 20 C. is 1.0236. Consequently, preparation of a mixture is unnecessary, and evaporation of part of the liquid does not affect the density of the residue. The cost is rather high, however. The xylene-chlorobenzene mixture is cheap, and, if kept carefully enclosed in a glass-stoppered bottle, it does not undergo appreciable change from evaporation.

The xylene-chlorobenzene mixture can be tested by known solutions of specific gravity 1.0235. As such, one may use sodium chloride, 3.36 Gm. per hundred cubic centimeters. A drop let fall into the organic mixture, in the manner described, should float within the organic fluid and neither rise nor fall.

Test Procedure for Plasma

The freshly drawn blood is mixed with heparin, 1 mg. per cubic centimeter of blood, or with oxalate, not more than 2 or 3 mg. per centimeter, and is centrifugated. The clear plasma is dropped from a pipet held about 2 cm. above the level of the fluorobenzene or xylene-chlorobenzene mixture. If the plasma specific gravity is below that of the fluid, the drop will rise rapidly to the surface, whereas if the drop has a higher specific gravity, it will fall rapidly to the bottom. Plasma just at the critical edema level will bob around if the tube is gently shaken, seemingly unable to decide whether to rise or fall. After a short time the drop flattens on the side or bottom of the tube and remains there as a record of its specific gravity.

---

[4] Fluorobenzene may be obtained from the Eastman Kodak Company, Rochester, N. Y.

[Reprinted from THE JOURNAL OF CLINICAL INVESTIGATION, September, 1932, Vol. 11, No. 5, pp. 871–895]

STUDIES ON THE EFFECT OF THE ACTION OF DIGITALIS ON THE OUTPUT OF BLOOD FROM THE HEART

I. THE EFFECT ON THE OUTPUT OF THE DOG'S HEART IN HEART-LUNG PREPARATIONS[1]

BY ALFRED E. COHN AND J. MURRAY STEELE

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, May 6, 1932)

A satisfactory solution of the problem concerning the effect of giving digitalis, especially on the volume output of the heart, but of other problems concerning its action, is still outstanding. Difficulties in the way of complete understanding depend, it seems, on the circumstances in which digitalis is administered. Among these are the species of animal which is studied and the state of the heart, whether healthy or diseased. The value of digitalis as a therapeutic agent, being on many accounts a constant source of concern, is viewed as being involved in these issues. Discussion is often confused, action of the drug and the beneficence of that action being regarded as the same things. This inference need not necessarily be correct. The pulse rate may be lowered, the blood pressure raised, the excretion of water unchanged, the volume output of blood from the heart either decreased or increased; the effect of not one of these actions can supply a clue, *a priori*, to whether giving this agent is advisable. In terms of no one of these functions can its value or benefit be described. The situation is different in an infectious fever. Barring the artificial lowering of temperature as a single effect without influence on the general course of the illness, defervescence is usually, and perhaps justly, regarded as a sign of value.

If the success of an agent cannot be measured in terms of a "test" of its efficacy, how can it be measured? Is it necessary to rely on the report of its effect by patients or is there something in the totality of their demeanor which permits the drawing of a relevant inference? In whatever way this phase of the matter is decided, an outstanding consideration is this, that a judgment is desirable which is not dependent on the measurement of some function, involved no doubt in the total action, but yet not characteristic of a result regarded as satisfactory. In the case of giving digitalis for example, patients may, conceivably, report "improvement" on taking the drug. Under these circumstances, if we associate with improvement increase, for example, in the volume output of blood from the heart and learn, unfortunately, that decrease has taken place, we face a dilemma; the output has decreased but the patient is better. To know the volume output is, for the present, an inadequate guide. The problem is not solved by turning from methods of analysis to others much coarser and not susceptible to detailed examination; that way would be the negation of method. Further study is needed to attempt to discover a form of analysis which tells us what we desire to learn, perhaps a method which measures the effect of an agent on the behavior of the entire individual. Knowledge may consist, obviously, in terms either of the whole or of one of its analyzed parts. But the balance between what the part can tell and what the whole describes is delicate; what must, it seems, not be upset is judgment of the whole, in favor of a part. Digitalis, in short, should be given if patients improve, even if, were this the case, the volume output of blood decreases as the result of taking it.

---

[1] A preliminary report of these researches was read before the Association of American Physicians. (Trans. Assoc. Am. Phys., 1931, xlvi, 71.)

The fruitful experiments of Harrison and Leonard (1) have made it clear that the volume output of blood from the heart can be reduced by digitalis. Important as this information is, suggesting, as it does, a new point of view from which to weigh the action of digitalis, a wide generalization based on this phenomenon seems inadmissible. For there may be, as has turned out to be a fact, a difference between hearts which are normal in size and hearts which are enlarged.[2] If an effect of digitalis is to decrease the size of all hearts, small as well as large, this result may still make a difference in the subsequent output of hearts initially normal and of hearts initially enlarged. But aside from size, there might be involved, in enlarged, diseased hearts, an additional factor. That this might be the situation was suggested in a report of experiments which confirmed those of Harrison and Leonard.

---

[2] The word enlarged is used, not to obscure the difference between dilatation and hypertrophy, but to suggest that of these conditions the one which is present is not always known. The two aspects of enlargement can, furthermore, not always be separated

In the experiments of Cohn and Stewart (2), decreased output of the normal heart as a consequence of the action of digitalis was described as a fact, associated with decrease in the size of the heart. The effect on output was ascribed to two factors, one on the size of the heart which was designated tone, and a second, an effect on contraction. Of the two, the effect on size predominated. This explanation Dock and Tainter (3) regard as unsatisfactory because it failed to take into account "all the known and established actions of the drug on the circulation and does not correlate the latter with factors determining cardiac output." They urged the view that in any judgment of the action of digitalis on the volume output it is not enough to study only the behavior of the heart; its effect on the blood vessels must also be estimated. The fact that in their experiments "a fall in venous pressure regularly accompanied the diminished cardiac output" they regard as "convincing evidence that the change in blood flow was a result of peripheral, and not cardiac, actions of the drug" (Conclusion 3) (3). Tainter and Dock (4) showed later that "the fall in right auricular pressure, after giving digitalis to dogs with the hepatic circulation intact, was due to diminished venous return flow, and ultimately to an accumulation of blood in the splanchnic or portal region as a result of obstructed hepatic outflow (hepatic vein constriction).

"This mechanism adequately explains the diminution in cardiac output after digitalis, since a diminished venous return must result in deficient cardiac filling. This conclusion agrees with that of our previous paper" (Conclusion 3) (4).

If both the heart and the peripheral circulation are, as is well known, affected by the action of digitalis, the problem in the case of normal hearts is this; how far is the effect of digitalis on the volume output exercised on the heart itself and how far on the amount of the venous return?

That there is a difference in behavior between enlarged hearts and hearts of normal size, and that this difference is of deciding importance, will be shown in these studies. What the relative share is of heart and periphery will also be discussed.

There is already evidence of the course of events in heart-lung preparations where a digitalis body is given to the heart in an enfeebled state. Hearts that were enlarged or failing have been studied by Anitschkow and Trendelenburg (5) who showed that by increasing the inflow (raising the venous reservoir in heart-lung preparations), the output, after the heart was made to fail was much greater than before if strophanthin were injected, and that corresponding to this effect the pressure in the right auricle fell. Bijlsma and Roessingh (6) noticed that in heart-lung preparations, when the heart was in process of dilating, it became smaller after giving strophanthin, though the resistance, the arterial pressure, and the volume output remained unchanged. Plant (7) also has shown that when strophanthin is given to a failing heart, poisoned previously during two to three weeks with phosphorus so that it is the subject of fatty degeneration, the volume output increased, the pressure in the inferior vena cava decreased; and the amplitude of contraction increased as did the blood pressure.

Here then are three sets of experiments, based on the study of failing hearts, all enlarged, in heart-lung preparations, in which increase in output went parallel with fall in venous pressure (Anitschkow and Trendelenburg, and Plant) and in which decrease in the size of the heart took place (Bijlsma and Roessingh). The effects were transient (Plant), the initial situation having been restored in 20 minutes.

The phenomena which are now reported were observed in hearts of heart-lung preparations, during a period of several hours until the time when complete failure of the preparation seemed imminent. As is well known, such preparations permit the control and measurement of several functions on which the circulation depends—the venous pressure, the volume output, the arterial resistance, the temperature. Obviously, hearts in heart-lung preparations cannot be regarded as fresh, or normal, or natural, due to the unwonted manipulations to which they have been exposed and also to the fact that they have been deprived of some of their extrinsic nerves. We do not, as a matter of fact, so regard them. Evidence that such hearts should not be considered fresh may be inferred from the fact that under the influence of digitalis, the minute output, unlike its effect in natural preparations and without regard to what ultimately will be held to be the mechanism, has not been seen to decrease, irrespective of the volume of the inflow.

For these experiments dogs weighing from 10 to 15 kilograms were used. Heart-lung preparations were made according to the general technique of Knowlton and Starling (8). The arrangements were varied, as will be pointed out, to suit the purposes of these experiments. Chloralose in physiological salt solution, neutralized by the addition of sodium hydroxide, served as anesthetic, and was injected intravenously usually in amounts of 0.1 gram per kilogram of body weight, but sometimes of 0.2 gram and rarely an addition of 0.5 gram. No morphine was given.

Blood for the experiment was secured from a second dog. Heparin, 5.0 mgm. diluted in 100 cc. normal salt solution, was injected first into a femoral vein; then, under local, novocaine, anesthesia, the blood required was withdrawn from the carotid artery. To complete the process of bleeding, light ether anesthesia was administered.

The dog lay in the lower half of a box. On opening the chest, ventilation was maintained by a Starling pump. When the preparation was complete, a lid closed the box which was then made airtight. Air in an amount sufficient to create the desired level of negative pressure was then removed by a large piston pump. To vary the degree of negative pressure and by so doing to secure motions of the lungs, air was allowed to enter the chamber from a 3 liter rubber bag (*RB*) through a valve (*V*, Fig. 1) the opening and shutting of which was managed automatically by the motions of the pump. The pressure in the box varied between −2 cm. and −4 cm. water during expiration (shutting of the piston) and between −12 cm. and −15 cm. during inspiration. In normal animals the range in the intact pleural cavity is between −2 cm. and −12 cm. The mechanism of the box was designed to simulate that of respiration in the normal chest.

Certain details in technique are described:

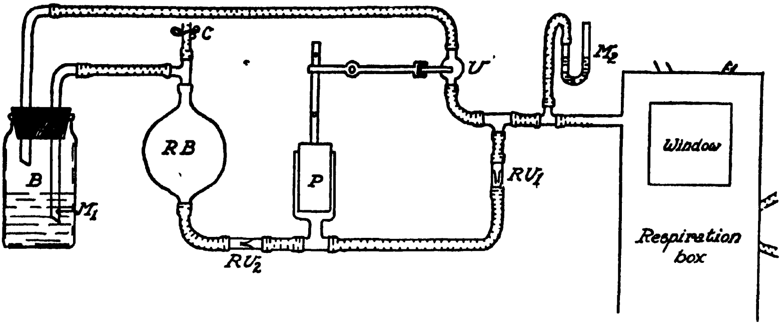


FIG. 1. This diagram shows the arrangement of the system used in artificial respiration in the heart-lung preparation.

The details are described in the text.

1. An upstroke in the pump (*P*) (capacity 4 liters) drew air from the box through the valve (*RV_1*), excluding at the same time, by closing the valve (*V*), that part of the circuit between valve (*V*) and valve (*RV_2*). A downstroke of the pump sent air through valve (*RV_2*), a rubber bag (*RB*), a water manometer (*M_1*), and so back to the box through valve (*V*), which had meanwhile been opened by the downstroke of the pump. A leak in the system was detected by ballooning of the bag (*RB*), distension of which could be relieved by opening a cock (*C*). The pressure in the system could be modified by the level of water in the bottle (*B*). A manometer (*M_2*) indicated changes in pressure in the box. By bubbling through water (in bottle *B*), the air in the system was kept moist.

2. When changes in the size of the heart were measured it was enclosed in a glass oncometer (Henderson) supplied with a rubber diaphragm, in which a hole was made, fitted snugly to the auriculoventricular groove (Figure 2). The oncometer was connected by pressure tubing with a volume recorder. At first a piston recorder (Huerthle) was used, next a cylindrical spirometer and finally, and also most satisfactorily, a Gad spirometer. The record of changes was inscribed on smoked paper.

3. The mean blood pressure in the brachiocephalic artery was recorded on the same smoked paper by a mercury manometer; that in the right auricle by a water manometer.

4. Peripheral resistance was provided in the usual manner (Knowlton and Starling (8)). Two resistance sleeves were placed in parallel in case of accident. The constancy of this system was checked by frequent readings of a mercury manometer.

5. The volume output was measured in a graduated cylinder, 50 ccm. or 100 ccm. being allowed to flow in on turning a three-way stopcock, the time required being taken by a stop-watch. Since the stop-watch was in an electric circuit the duration of the period was recorded on the smoked paper.

6. The height of the head of pressure of the blood flowing into the heart was kept constant. The reservoir was a flexible rubber bag immersed in a large jar of water (9). Through a tube of large bore (1″) water in the beaker was displaced and passed freely to a bottle suspended by rubber, when blood in the reservoir exceeded a previous volume; conversely, decrease in the volume of blood in the reservoir invited a return flow of water into the beaker. The level of fluid once set remained, therefore, constant. By a rack and pinion, the height of this unit was easily adjusted.

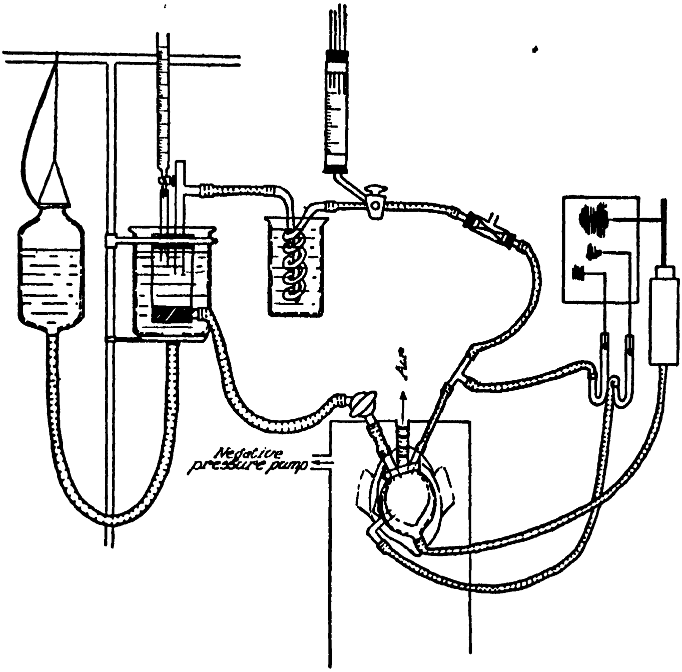


Fig. 2. This diagram shows the arrangement of the system used for the circulation of blood in the heart-lung preparation.

7. A stopcock, with large bore, was placed between the reservoir and the right auricle. A dial showing divisions mounted on the housing, and a pointer sealed to the cock, permitted the repetition of a given setting. The inflow could accordingly be reduced, simulating constriction of the veins.

8. A thermometer, placed in the cannula inserted in the superior vena cava, indicated the temperature of the blood (not shown in the figure).

9. A solution of glucose and insulin flowed continuously into the venous reservoir from a graduated burette.

10. From a pet-cock, placed in the circuit just before the venous reservoir, blood was taken for analysis, and substances, such as digitalis (Digitan, Merck) were injected (not shown in the figure).

11. Time was recorded on the smoked paper at intervals of 1 second and 10 seconds.

PROCEDURE

Experiments of three types were performed. In the *first*, the ventilation was managed by the method of negative pressure—and without an oncometer about the heart. Although the degree of negative pressure which was exerted was comparable to that in the normal chest, when the bony thorax was divided and the edges were held apart, the lungs fell away from the heart and failed, therefore, to give it that support which normally protects it from the full force of negative pressure. How great this effect may be is observed in the record in which fluctuations due to this influence are clearly observed in the records of arterial and venous pressures. The latter ranged between −3 cm. and +1 cm. water. The *second* type of experiment was like the first except that an oncometer was placed over the heart. The result of this arrangement seemed to approach more closely that in a normal intact chest. The venous pressure ranged between +2 cm. and +5 cm. water. In this type is included a sub-group (5 experiments) in which was studied the effect of varying the volume of inflowing blood. In a *third* type, ventilation was positive, the Starling pump being employed. An oncometer was again fitted over the heart. This arrangement exhibited the least effect of respiration on the arterial and venous pressures and on the size of the heart. The venous pressures were the highest observed, ranging from +5 cm. to +8 cm. of water. They were lowest in the first group.

The arrangements employed, taken together, provided a range of venous pressures running from a level above to one below normal. Though the natural situation was absent, it has been possible, nevertheless, to test the action of digitalis under very varying conditions, within the range of which, many, even the normal ones must have been present. Though different in degree, the effects of giving digitalis tended, whatever the arrangements, in the same direction.

The results in each type of experiment are illustrated by describing one example in detail.

When the heart was not enclosed in an oncometer, it was the usual experience (Figure 3) to observe on transition to negative pressure that, due no doubt to the pull exerted on the heart, for this effect did not appear when it was protected by an oncometer, pressure in the right auricle fell abruptly. At the same time the output of blood per minute and per beat from the heart increased slightly. Under negative pressure, the pressure in the right auricle remained practically stationary for about one hour. For about another half-hour it fell slightly, while the volume output decreased, also slightly. Then the heart began to fail, as is shown by the rise in pressure in the right auricle and in the sharp fall in volume output. The temperatures, meanwhile, underwent no important change. The arrangement of the "venous" reservoir remained constant throughout the experiment, the height being 7.0 cm.; it seems doubtful whether the changes in the rate of the heart were influenced by the alterations in temperature which occurred. The heart was permitted to fail until, after two hours, the output per minute fell to about 225 ccm. Synchronously, the pressure in the right auricle rose slightly, but distinctly. Digitalis (in the form of digitan, 0.25 gram[3]) was then injected. One

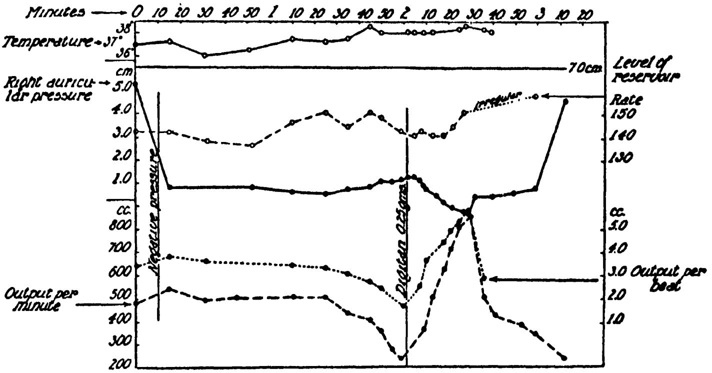


FIG. 3. The curves are shown of the course of events in experiment number 44. The details are given in Table 1.

---

[3] This dose was calculated on the basis of 0.023 gram per kilogram. An amount having been calculated, as being suitable for intact animals, a smaller dose was given, taking the nature of the preparation into consideration. Later, doses still smaller were given.

half-hour after injection, the output increased as much almost as four hundred per cent, and was greater by about fifty per cent (550 ccm. to 825 ccm.) than the output just after the preparation was placed under negative pressure. During this period the pressure in the right auricle decreased to a point lower than during the early, steady period, falling so low indeed, that negative pressure was recorded; the ventricles were ejecting blood at least as fast as it was being supplied. An excess in the reservoir prevented exhaustion of the supply.

TABLE 1

March 5, 1931. Dog Number 44. Weight of Dog—10 kgm. Weight of Heart—88 Grams. Anesthesia—Chloralose—1.2 Grams

Time	Temperature	Arterial pressure	Right auricular pressure	Rate	Volume in reservoir	Output per minute	Output per beat	Changes in heart volume	Glucose burette level	Blood sugar
a.m.	*°C.*	*mm. Hg*	*cm. H_2O*	*per minute*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*mgm. per 100 cc.*
10:52	Operation began									
11:30	Circulation turned over. Level of venous reservoir set at 7.0 cm.									
p.m.										
12:00	37.0	100	+5.3	142	625	480	3.4		1.1	93
12:10	Changed to negative pressure respiration									
12:15	37.2	106	+0.8	142		540	3.8			
12:31	36.0			138		490	3.6			
12:58									5.8	111
1:25	37.2	108	+0.5	150		500	3.3			
1:45	38.5	110	+0.8	150	525	400	2.7			
1:50	38.0	110	+1.0	148		350	2.4			
1:55			+1.0			270				
1:59		106	+1.1	142		230	1.6		24.4	172
2:03	Digitan 0.25 gram		+1.2							
2:06	38.0	106	+1.1	142	500		2.5			
2:09	38.0		+0.85	142		360				
2:10			+0.7				3.6			
2:14	38.0		+0.4	140		500				
2:18			+0.1	140		620	4.4			
2:22			−0.1	144		710	4.9			
2:25	38.2	110				800	5.3			
2:27	38.5		−0.3	150	490	850	5.4		34.3	202
2:36	38.2			Irregular		500				

Too much digitalis, obviously, was injected, for the rate of the heart, having been steady for almost twenty minutes, increased rapidly; the rhythm then became quite irregular. The heart was irretrievably intoxicated. The output fell rapidly and in forty minutes was as small as just before digitan was given. Toward the end of the experiment, pressure in the right auricle rose.

The course of events in this experiment is clear enough. At first the preparation was sufficient. When it was near the point of death, the administration of digitalis revived it. Its effectiveness then was greater than it had been throughout the period of observation. Clear as is the demonstration that a dying heart can be revived, it was impossible to estimate what changes took place in its size, nor was it clear whether the heart was enlarged when digitalis was injected.

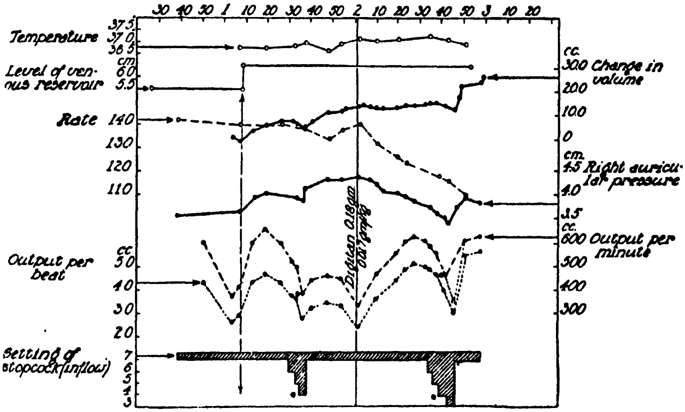


Fig. 4. The curves are shown of the course of events in experiment number 59. The details are given in Table 2.

The next experiment (the *second type*) gives information on this point. The heart was enclosed in an oncometer. And it gives information also on another point. The stopcock, already described, was inserted in the tube leading to the right auricle. When the box in which the dog lay was subjected to negative pressure, that fall in pressure in the right auricle which took place when the heart lay bare, now, when an oncometer was fitted to it, failed to occur.

After about thirty minutes (Figure 4), the level of the venous reservoir having been set at a height of 5.5 cm., the volume output per

TABLE 2

April 20, 1931. Dog Number 59. Weight of Dog—10.5 Kgm. Weight of Heart—90 Grams. Anesthesia—Chloralose—1.15 Grams

Time	Temperature	Arterial pressure	Right auricular pressure	Rate	Volume in reservoir	Inflow setting	Output per minute	Output per beat	Changes in heart volume	Glucose burette level	Blood sugar
a.m.	*°C.*	*mm. Hg*	*cm.* H_2O	*per minute*	*cc.*		*cc.*	*cc.*	*cc.*	*cc.*	*mgm. per 100 cc.*
11:18	Operation began										
11:58	Circulation turned over. Level of venous reservoir set at 5.5 cm. and changed at 1:12 to 6.5 cm.										
p.m.											
12:50		102	3.5	142	400	7	600	4.3	0	7.9	112
1:06	36.8	108	3.6	140			410	2.9	−1.5		
1:12		108	3.9				600	4.3	+3.0		
1:18	36.8	110	4.0	140	400		660	4.7	+5.5		
1:25				140			600	4.3	+5.0		
1:28						6					
1:30	36.9						520	3.7	+7.0		
1:31						5					
1:32			3.9				500	3.6	+6.0		
1:33						4					
1:35			3.85	138			380	2.8	+4.5		
1:35+						7					
1:36	37.0		4.1				440	3.2	+7.0		
1:46	36.7	114	4.3	134			460	3.4	+11.0		
1:52	37.0		4.3	138			450	3.3	+11.5	19.6	141
2:00	37.2	114	4.35	140			330	2.4	+13.5		
2:01	Digitan 0.18 gram										
2:08	37.1		4.2	132	375		475	3.6	+13.0		
2:17	37.2	114	4.0	126			560	4.4	+13.0		
2:21		114	3.95	124			610	4.9	+13.5		
2:25		114	3.85				630	5.1	+14.0		
2:30							610	5.0	+14.5		
2:31						6					
2:32	37.3		3.70				590	4.9	+15.0		
2:33						5					
2:35			3.60				560	4.7	+15.0		
2:36				118		4					
2:39			3.50				460	4.0	+14.0		
2:40						3					
2:43	37.2		3.40	116	125		350	3.0	+12.5		
2:44			3.7			7			+17.5		
2:46					325 (200 cc. blood returned)						
2:48	37.0	116	3.9	110			610	5.7	+22.0	29.0	174
2:55		116	3.8	110			630	5.7	+24.0		
2:56	Rubber for peripheral resistance broke										

minute, then about 600 ccm., fell in the next ten minutes to 380 ccm. The level of the reservoir was then raised to 7.0 cm., where it remained throughout the experiment. The output rose to about 630 ccm., higher than it had been before. The volume of the heart increased; the pressure in the right auricle rose. The behavior of the heart having been satisfactory for fifteen minutes, the stopcock in the inflow tube was closed bit by bit. Output per minute and per beat, pressure in the right auricle and the volume of the heart, all promptly and expectedly decreased, and decreased the more, the more nearly the stopcock was shut. After ten minutes the stopcock was again opened wide. At once, output, volume of the heart and pressure rose, but not to the initial height. The heart seemed no doubt to have been deteriorating, as is shown by its increasing volume. After another twenty minutes the preparation began seriously to fail, the output decreased to about 325 ccm., the pressure in the right auricle rose, and the cardiac volume increased still further. The temperature of the blood and the rate of the heart beat remained meanwhile sufficiently steady. Digitalis was now injected. In ten minutes a substantial increase in volume output occurred and in thirty minutes the amount put out at one hour had almost been reached. The volume of the heart, which before injection had steadily been increasing, now ceased to do so; on the contrary, it became a little smaller. The pressure in the right auricle promptly declined, falling to a level lower than when the output was approximately the same as now. As the rate of the heart beat fell, the output per beat increased, so that the curve describing this function continued roughly parallel to that of the output per minute. There was, nevertheless, no increase in the heart's size. Half an hour after the injection of digitalis, the maneuver with the stopcock was repeated. The course of events noticed before took place again. The minute output and output per beat declined, the pressure fell, the heart's volume decreased. When the stopcock had been shut to a point identical (marked by asterisks on the stopcock and also on "output per minute" curves) with that before injection, it is apparent that, although the minute output just before closing the stopcock was approximately alike on both occasions, now, after the injection of digitalis, the same setting resulted in a smaller effect than before—the minute output did not decrease so much. It was greater in fact by about 80 ccm., an amount equal to 12.0 per cent of the highest output. A tighter shutting of the stopcock had, of course, a correspondingly greater effect, as the dotted line at 2.25 shows. Together with the change in minute output, corresponding changes in the volume of the heart and in right auricular pressure took place. The rate of the heart beat continued to fall so that the curve describing output per beat is higher than before. When the stopcock was opened again, the output increased to its former amount, the pressure in the right auricle also returned, but showed no disposition to rise further, evidence of the good state of the preparation. Shutting the stopcock seems, however, further to have damaged the heart for now in order to maintain its output, it dilated sharply.

This experiment shows that: (1) Giving digitalis when the heart was in a state of acute failure, revived it, so that the initial minute output was restored. (2) Although before the administration of digitalis the heart was continuously dilating, afterward, further deterioration was arrested, until decreasing the inflow again, by shutting the stopcock, still further injured the heart. (3) Decreasing the inflow by means of the stopcock exerted a smaller effect on the minute output, after giving digitalis than it had before. Exactly how this difference is to be "explained" is not apparent. Greater contractile efficiency, decrease in rate, balanced by greater output per beat, made it possible to receive and to expel greater volumes of blood, the reservoir arrangements having remained unchanged. That the volume of flow through the heart was greater is shown by the fall of pressure in the right auricle. (4) The effect just described is characteristic of this preparation, free as it is of hepatic and all other veins save the pulmonary. The failing heart, it appears, is able, despite the decrease in inflow brought about by constriction of the veins, simulated by shutting the stopcock in this case as much as about 40 per cent, to expel more blood at the same setting under the influence of digitalis than before its administration. Inflow is in short a function not solely of the degree of constriction of the inflow tract; the state of efficiency of the heart, its ability to receive and to eject blood, is another relevant factor.

Still other experiments (the *third type*) were performed in dogs in which respiration was maintained by positive pressure, a Y-shaped cannula being fixed in the trachea, the two limbs of which were connected with the inlet and outlet tubes of a Starling pump, and in which an oncometer was fitted over the heart. In an illustrative experiment (Figure 5) failure of the heart was signalled by a fall in volume output from 700 ccm. to 410 ccm. per minute. The heart dilated, the venous pressure rose. Important changes did not take place in the pulse rate or in the temperature. When digitalis was injected, the minute output rose to almost its original level. There was prompt and striking decrease in its volume, the decrease continuing for a short time after the maximum output was attained. In all probability, the pericardium being open, the heart even then was relatively dilated, in comparison with its original, natural size. The change illustrates the fact, however, that, when heart muscle fibers are stretched more than a certain length, shortening does not, as is the case of normal hearts, decrease but rather increases the minute output. Coincident with the rise in output, occurred a distinct fall in right auricular pressure. The rate of the heart beat also fell but only six a minute.

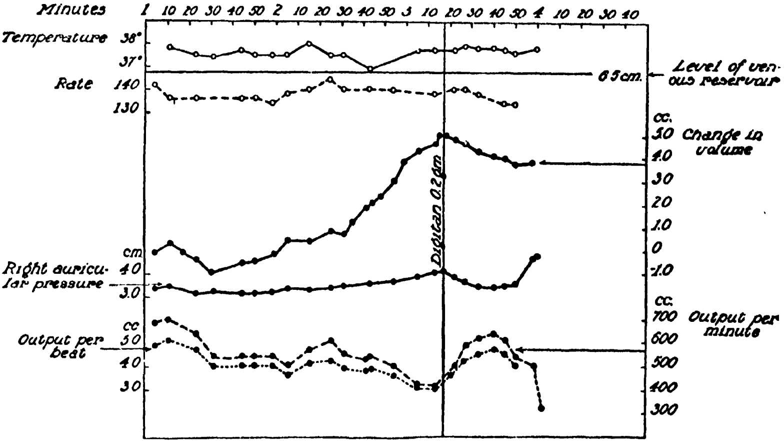


FIG. 5. The curves are shown of the course of events in experiment number 65. The details are given in Table 3.

Propelling blood against more than an atmosphere of intrapulmonary pressure did not in this experiment interfere with the ability of

TABLE 3

June 10, 1931. Dog Number 65. Weight of Dog—11.5 Kgm. Weight of Heart—109.5 Grams. Anesthesia—Chloralose—1.25 Grams

Time	Temperature	Arterial pressure	Right auricular pressure	Rate	Volume in reservoir	Output per minute	Output per beat	Changes in heart volume	Glucose burette level	Blood sugar
a.m.	°C.	*mm. Hg*	*cm. H_2O*	*per minute*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*mgm. per 100 cc.*
11:28	Operation began									
p.m.										
12:21	Circulation turned over. Level of venous reservoir set at 6.5 cm.									
1:04		106	3.4	142	330	690	4.9	(100 cc. blood added to reservoir)		
1:07					620					
1:11	37.8	106	3.5	136		700	5.1	+0.4	0.1	82
1:23	37.5	104	3.2	136	700	640	4.7	0		
1:31	37.4	104	3.25			540	4.0	−0.9		
1:44	37.7	104	3.2	136	600	540	4.0	−0.5		
1:50	37.5	104	3.2	136		540	4.0	−0.5		
1:58	37.5	104	3.25	134		540	4.0	−0.1		
2:05	37.5	104	3.4	138		500	3.6	+0.5	38.5	402
2:15	38.0	106	3.35	140		570	4.1	+0.5	(Blood noted in pericardium)	
2:25	37.5	106	3.4	144		610	4.2	+0.9		
2:31	37.5	106	3.5	140		550	3.9	+0.8		
2:41						530	3.8	+1.8		
2:43	36.9	106	3.6	140		540	3.8	+2.1		
2:54		106	3.7	140		500	3.6	+3.1		
3:05	37.7	106	3.9			420	3.0	+4.4		
3:13	37.7	105	4.1	138	200	410	3.0	+5.1		
3:17	Digitan 0.2 gram		4.15						48.8	402
3:22	37.7	106	3.9	140		500	3.6	+4.9		
3:27	37.9	106	3.7	140		590	4.2	+4.7		
3:33	37.8	106	3.5	138		620	4.5	+4.4		
3:40	37.8	106	3.45			640	4.7	+4.2		
3:45	37.7	106	3.5	134		610	4.5	+4.1		
3:50	37.6	106	3.6	134		540	4.0	+3.9	52.3	436
3:58			4.7			500	?			
4:00	37.8		4.8			310				

the heart to increase its output when digitalis was administered. It is striking, furthermore, how great were the concomitant effects on the size of the beat and on the pressure in the right auricle. Despite decrease in volume, the output was increased to so great an extent that it was able to receive blood and to propel it onward toward the lungs so competently as to lower the pressure in the right auricle very considerably.

Twenty-three experiments in all are reported. In one-half the final effect of the action of digitalis was to decrease the rate of the heart, though in one-half of these, the fall was preceded by a slight rise. In two experiments only did the rate increase promptly and permanently. In the remainder it remained practically constant. These statements refer only to rates during periods of normal cardiac mechanism. As terminal phenomena, paroxysms of ventricular tachycardia were frequently observed, and on a few occasions recoveries from paroxysms, in early stages. In no instance did the rate of the heart beat change sufficiently so that alterations in it could be regarded seriously as responsible, during the period of the action of digitalis, for altering the cardiac minute output.

Five experiments were valueless for the purpose of securing measurements of venous pressure, of cardiac output, and of changes in size of the heart. In one case (Number 63) clots formed in the circulating blood; in two others (Numbers 52 and 60) large hemorrhages took place in the cardiometer; and in two additional ones (Numbers 55 and 57) ventilation under negative pressure failed. The two cases last mentioned are, however, not without interest. Although they cannot yield measurements comparable with those found in other instances, they do, nevertheless, suggest reasons which explain the failure of the action of digitalis (Table 4).

The remaining eighteen experiments (Table 4) were sufficiently satisfactory to permit deductions to be made from the measurements. In all of them, the temperatures and the rate of the heart beat were approximately constant; these factors need, therefore, not to be taken further into consideration. The venous reservoir remained at a constant level after it was definitely set. Since the pressure level of the "peripheral resistance" varied within slight limits only during the course of an experiment, changes in brachiocephalic blood pressure did not occur. For these reasons, the following six variable factors only require study: (1) the minute output from the heart, (2) the size of the heart, (3) the pressure in the right auricle, (4) the condition of the heart, (5) the condition of the lungs, and finally (6) the condition of the blood which is in turn dependent on the state of the lungs and the rate and volume of the blood flow. Since experiments showing gross changes in the lungs and in the blood were not further studied, the last two factors (5) and (6) may be ignored. There remain, therefore, only four variable factors. The first three can all be measured and since in fact they are dependent on the condition of the heart, measurement of them describes in turn the state of this organ. Changes in these measurements depend, therefore, upon changes in the heart. It should be possible, consequently, by comparing measurements made before giving digitalis with others made afterwards, to describe certain of the effects for which the administration of this drug is responsible (Table 4). Descriptions of three typical courses of events have been given already. The following description may serve, therefore, as a general summary.

When the pressure in the right auricle rose slowly and steadily, the volume output per minute and per beat decreased, and the size of the heart simultaneously increased. A cardiometer was employed in ten only of the experiments. In one case (Number 60) a leak of air invalidated the results. But in the other nine instances, measurements made it certain that the hearts dilated. In the eight cases in which an oncometer was not used, it seems certain also that the hearts increased in size.

The effect of the administration of digitalis was prompt and definite as measured by the three criteria on which reliance was placed: the minute output, having fallen, rose; the pressure in the right auricle, having risen, fell; the heart, having dilated, contracted. These results were noticed in fourteen of the eighteen satisfactory experiments. Of the remaining four, one (Number 57) only showed no effect, but in the other three (Numbers 49, 52, 55) the effects were either slight or their progress having begun was promptly halted so far as concerns reduction in size of the heart and rise in right auricular pressure, though in all three the output exhibited a slight rise.

DISCUSSION

These experiments, it seems, supply evidence which shows that hearts which are obviously failing, in that, although constantly in-

TABLE 4

A Summary of the Individual Experiments in the Three Groups

Dog number	Change of temperature	Rate	Right auricular pressure	Volume output per minute	Volume output per beat	Change in volume of heart	Duration after digitan	Dose	Constriction of inflow
	°C.	*per minute*	*cm. H_2O*	*cc.*	*cc.*	*cc.*	*minutes*	*grams*	
Group I. Negative pressure, no oncometer									
36	0	128‡ 134	+0.4 −3.0	290 600	2.4 3.4		30	0.5	
37	0	138 142	−1.2 −4.0	200 470	1.4 3.4		20	0.3	
38	0	138 114	+ −	90 240	 +		40 10	0.3 0.1	
39	0	146 126	+2.2 −1.0	240 920	 +		30	0.3	
41	0	144 114	+0.8 −2.0	200 510	 +		55	0.25	
42	0	118 110	−3.6 −4.6	200 420	 +		65	0.25	
44	0	140 150	+2.4 −0.8	230 850	1.6 5.7		70	0.25	
45	0	166 186	 −?*	610 760**	3.6 4.1		100	0.25	
Group II. Negative pressure, with oncometer									
49	+1.2	134 138	+20 +20	 −?*	 −?*	 †	37	0.25	
50	−1	140 168	+4.4 +2.2	690 1070	4.6 6.1	 −1	44	0.25	
51	0	154 156	+7.4 +6.0	600 700**	 +?	 −8	35	0.25	
52	0	136 136	+5.2 +5.2	 −?*	 ?	 ?	35	0.25	

‡ Whenever two figures appear in a space the upper represents the value recorded immediately before the administration of digitalis, the lower the maximum effect of the drug.

* Continual decrease during the experiment.

** Continual increase during the experiment.

† Oncometer leaked.

TABLE 4—*Concluded*

Dog number	Change of temperature	Rate	Right auricular pressure	Volume output per minute	Volume output per beat	Change in volume of heart	Duration after digitan	Dose	Constriction of inflow
	°C.	*per minute*	*cm. H_2O*	*cc.*	*cc.*	*cc.*	*minutes*	*grams*	
53	0	136	+7.8	310		§	24	0.20	
		128	+7.2	500	+				
54	−0.7	120	+8.9	290	2.6	§	87	0.15	−80 cc. −19.5 per cent
		116	+9.0	470	4.4				No reduction
55	+1.7	126	+4.7	260			36	1.5	
		112	+4.8	270	?	+?	5	0.1	
56	0	140	+11	300			73	0.2	−270 cc. −45 per cent
		104	+10.6	580	+	−5			−210 cc. −36 per cent
57	0	134	+4.0	340			35	0.18	Reduced
		134	+4.0	300*	−?	−***			Not done
59	0	140	+8.7	330	2.4		56	0.18	−220 cc. −36.7 per cent
		110	+7.7	630	5.1	−§			−150 cc. −26.0 per cent
60	0	134		360			25	0.18	Reduced
		132	+?**	300*	−?	−?†			Not done
Group III. Positive pressure, with oncometer									
63	−1.5	134	+9.0	320		†	34	0.18	
		130	+8.0	370	+?				
64	0	148	+9.8	390			48	0.2	
		150	+9.0	460	+	−6.0*			
65	0	138	+8.3	410			45	0.2	
		134	+6.9	640	+	−1.2			
66	0	128	+14.2	300			63	0.2	
		120	+11.7	590	+	−3.1			

§ Further increase in size postponed for varying periods of time although no decrease occurred.

*** After revival by positive pressure.

creasing in size they are expelling ever smaller volumes of blood can, nevertheless, as a consequence of receiving digitalis, deliver greatly increased quantities. Since there are no blood vessels, except the bronchial and pulmonary vessels, nor any other structures in the preparation than the lungs, no other mechanism can be responsible for this occurrence than the heart itself. The heart can apparently become smaller and at the same time more efficient in increasing the volume of blood which it expels. That an effect of the action of digitalis is exercised on heart muscle is well known, and also that this effect may consist at one stage in increasing the degree of its systolic contraction. Experiments have shown in fact that "improvement" in the behavior of the heart takes place also in damaged and feeble hearts. But these experiments supply evidence not only that damaged and feeble hearts can be so influenced but also that hearts which are dilated can. And they show, furthermore, that when they expel greater volumes, they may at the same time diminish in size. Smaller hearts manage, in short, to increase the volume output.

This situation is different from that in hearts which, because obviously undamaged, are of normal size and in certain other hearts which are damaged but are not dilated, even as the result of forced exercise (Harrison and Leonard (1), Cohn and Stewart (2)). The hearts which were studied in the experiments now reported were plainly not fresh hearts. To draw inferences from their use, as if they were, would be unjustified. But the point at issue has not to do with whether they were fresh or old, but essentially with whether the effect of digitalis is exerted in a significant fashion on the heart muscle, and with what that effect is. If its action on enlarged hearts, dilated or hypertrophied, is admitted, but an effect on normal sized hearts is denied, a point in the process of lengthening must be found at which the muscle *begins* to be susceptible to the action of the drug. There may be such a point, but its presence has not, so far, been the subject of investigation. The volume outflow may under definite circumstances reflect the action of other influences. But to arrive at a complete description, it is not enough to study only the peripheral action of digitalis. Its cardiac action has consequently been investigated in detail. In other experiments, to attempt to ascertain what effect on the net result might be contributed by an action on the peripheral circulation, especially in the portal and hepatic regions, the stopcock was placed in the inflow tract to the heart. Reference will be made to this type of experiment later.

The decrease in volume output which has been noticed was thought to result from the tonic effect of digitalis upon the heart (2), in consequence of which its size diminished so much, that a large or even a usual volume of blood could not be received and, therefore, could not be ejected. Dock and Tainter (3, 4) believe that an explanation of this sort must be rejected because it fails to take into account the behavior of the rest of the circulatory apparatus which, as they think, is simultaneously affected. They have drawn attention (4) to a mechanism in the hepatic veins by which, under the influence of digitalis, the lumina, the walls containing smooth muscle which contracts, diminish and prevent the return of blood to the heart (10) because it is dammed back into the intestinal cavity. The decrease in output from the heart results accordingly not from a primary effect upon the dimensions of the heart but from a secondary one due to constriction of the hepatic veins. The heart becomes small, not because of the effect upon it of the action of digitalis but because it can not be filled.

Now the vessels which transfer blood from the descending aorta to the inferior vena cava may be said to consist of a main circuit, returning blood chiefly from the kidneys, the pelvis, and the legs; and a shunt which conveys blood to the intestinal viscera, chiefly to the spleen, the pancreas, and the intestines, and which then passes through the liver, entering the inferior vena cava finally through the hepatic veins. The blood flowing into the right auricle is derived both from the superior and the inferior vena cava and the vena azygos. The portion which the hepatic veins contribute to the inferior vena cava is, as Grab, Janssen, and Rein have shown (11) approximately 50 to 60 per cent or about one-quarter of the amount which flows to the right auricle. On occasion this amount may be augmented to about 80 per cent or about one-third of the total.

It seems to be a fact that not only individuals, but species vary considerably in the amount of smooth muscle which the walls of the hepatic veins contain. On this, when present, digitalis acts. In consequence of this action, the smooth muscle contracts and by so doing narrows the lumen so that blood is dammed back in the portal system. That this result occurs, especially in dogs, is known because the pressure in the portal system rises but the extent of the pooling, important in the mechanism only if great, is not known. But, as recent investigations have shown (12) it is dogs especially which exhibit this anatomical peculiarity. The hepatic veins of cats, for example, and of human beings fail to exhibit conspicuous amounts of the smooth muscle. Whatever may be the case regarding the mechanism in question in dogs, it cannot be operative in human beings because plainly it does not exist in them to a significant degree.

But in a given dog the possibility of the existence of this mechanism remains, capable of reducing the return flow to the heart to an extent so great that the total volume inflow and in consequence the size of the heart are reduced on this account to the extent that observation shows them to be.

The anatomy of the hepatic veins themselves requires, however, close scrutiny. They can, as has been said, be responsible for conveying 50 per cent of the blood which flows back into the inferior vena cava. They are in short each, right and left hepatic veins, large structures. Their united circumference exceeds that of the inferior vena cava (13). In order to be effective in shutting off from the volume of blood returning to the right auricle as much as a quarter, the lumina of the two vessels must be reduced to zero—must be completely obliterated. What the facts are is not known. The muscle in the walls of the hepatic veins, though abundant, seems scarcely capable of so powerful an action. But if they cannot close wholly they may conceivably close as much as 50 per cent. If they manage this, they deprive the inflow to the auricle of one-eighth (12 per cent) only of its total volume. And even if they succeed in bringing about this result, the diminution should be incapable of explaining the facts found in earlier experiments (2*a*). The results that were found in those dogs were striking. The outflow decreased not 12 per cent but in amounts varying between 34 and 68 per cent (with one exception in which it was only 14 per cent Table 5A). These are the consequences of giving digitalis to dogs, the hearts of which were normal. When similar experiments were attempted in the dogs in which the hearts were enlarged (hypertrophied) (2*b*) as the result of long standing damage to the mitral valves, the reduction in output was at least as great as in the normal ones and in some cases even greater (Table 5B). To refer the explanation of decreased outflow to decreased inflow—due to contraction of the hepatic and mesenteric veins seems, therefore, incorrect.

But this explanation of the mechanism of diminished outflow seems unlikely for other reasons as well. The effect of the action of digitalis upon the heart endures for days. There are records (Cohn and Stewart) of the persistence of the action as long as 10 days; and durations of 3 days are not uncommon. In human beings all the effects have often not disappeared even after 21 days. In comparison with durations as long as these, those reported on the length of time the effect of the action of digitalis has been observed on the hepatic veins must be regarded as evanescent. Unfortunately no example is shown (Tainter and Dock) in which the effect persisted longer than 25 minutes or, when digitalis and strophanthin were given in succession, for longer than 40 minutes. The observations were then terminated.

TABLE 5

*The Effect of Digitalis on the Cardiac Output in Dogs with Normal Hearts (A) and with Enlarged Hearts, the Result of Artificial Valvular Lesions of Long Standing (B)**

A		*B*	
Dog number	*Per cent decrease*	*Dog number*	*Per cent decrease*
265	66	162	58
257	38		67
258	48		32
253	46	161	62
254	45		31
252	35	158	75
251	53		65
255	34		47.4
264	14	155	44
259	50	171	83
261	68	90	23
263	47		

* The data in this table are taken from the tables in papers by Cohn and Stewart (2*a* and *b*).

If rise in pressure in the portal system is taken as the duration of the action of these drugs on the hepatic veins, 5 to 12 or 15 minutes seems to be the maximum period. If the criterion is fall of pressure in the right auricle, this effect did not occur in Dog 1, began to disappear in Dog 2 after 14 minutes, and in Dog 4, after giving strophanthin, had regained its initial level in 20 minutes. It lasted somewhat longer, for 2 hours, in Dogs F4 and F5, when the records stop. The constrictor action of digitalis on the hepatic veins occurs, no doubt. When it fails to do so in Dog 1, the hepatic veins may be poor in muscle. Interesting though this action is, it seems to be transient. A point of importance should be made on the matter of the duration of effect. If, as between effect on heart and on hepatic veins, that on veins persists, it is essential to know in general how long this action may be. That on the heart is known to last for several, perhaps for many days. If that on the veins is as long, the action of both structures must contribute to the final result; if not, that on the veins possesses a slight influence only. The evidence concerning the effect on the veins so far assembled is meagre.

In intact dogs (2*a* and *b*) the effect of injection was studied 2 to 3 hours after injecting digitalis, except in two instances when the times given are 1½ to 1¾ hours. And the persistence of the action is not minutes but 1 to 3 to 10 days. If, as seems to be the fact, the hepatic veins become narrow, the duration of this influence of digitalis is so brief that there must be hesitation in attributing to this effect of the drug, responsibility for the long persisting decrease in outflow, a decrease which does not begin to be effective, or at all events did not begin to be studied, until 2 hours (2*a*) after injection, and which persists for many days. The hepatic mechanism is plainly non-existent in any important sense. If objection is made to ignoring the short duration of the effect of digitalis on the heart in heart-lung experiments and to insisting upon it in connection with experiments on the hepatic and splanchnic veins, it may be pointed out that the two experiments differ. Those on the heart come to an end not because the action of digitalis ceases, but because it proceeds often to still greater and graver damage. The effect on the veins ceases because in all probability it is overshadowed by the results of trauma. Whether its action persists, nevertheless, is unknown. In intact animals the situation is different; the duration of the effect is longer but it has not been observed beyond two hours.

To test the relation of low venous pressure to decreased minute output, those experiments were performed in which the calibrated stopcock was inserted in the inflow tract. The pressure in the right auricle rose, as has been described, when the heart began to fail; it fell when the heart revived. There can be no question in these experiments of a venous (hepatic) mechanism; there were no veins. Nor can there have been any change in the volume of blood to which the heart had access; the amount, due to the arrangement of the experiment was always the same.[4] If a change in pressure took place, as it did, the behavior of the heart alone must have been responsible. And this change, a fall in pressure in the right auricle, it was able to bring about even when, and this point is not without interest, a decrease in its size took place. Decrease in the return flow is not the condition, obviously, which is responsible either for decrease in the size of the heart, for the outflow increased, or for the fall in pressure in the right auricle, because there was no decrease in the volume of blood ready to flow in.

A further test could be made of the possible influence of constricted hepatic veins on the net result of giving digitalis, so far as output is concerned. It has already been suggested that the maximum effect likely to take place as the result of constriction of the hepatic veins due to giving digitalis is a reduction in inflow of about 12 per cent. If the veins were shut entirely the effect, an unlikely occurrence, would be 25 per cent. When the heart in the experiments now reported began to fail, the cock to which reference has been made was partially shut, so that volumes less than the initial ones were available to the heart. Reductions in caliber greater than 10 per cent effected a reduction in output but, and this is the point, the reduction in output at identical settings of the stopcock was greater in the undigitalized failing, than it was in the digitalized, heart. Giving digitalis may constrict the hepatic veins so that the output from the heart is diminished but, as this experiment shows, this is a consequence depending not simply on the degree of constriction of the veins but on the difference in the heart after giving digitalis. The dilated failing heart can, from the same reduced inflow path, manage a greater output; and in the intact animal when the entrance pathway is reduced to 75 to 87 per cent, the heart can still deliver a greater volume than before.

---

[4] Those experiments in which the volume of the inflow is varied by changing the level of the reservoir are obviously different. In these the head of pressure remains constant.

But for the normal heart the restriction in inflow cannot, as has been shown, represent the facts. Proof that this must be the mechanism on which decreased size of the heart and decreased output depend has been believed (4) to depend on the low level of venous pressure. Evidence beyond that found in these experiments, which describe the course of events in failing hearts, to show that low venous pressure and small size together with diminished output, as is found in normal hearts, are not in necessary relation, is supplied in a study of the action of digitalis in normal human beings. It is undesirable to anticipate the report (14) which describes such observations, but mention may be made now of the fact that, in them, measurement of the venous pressure fails to show, on the assumption which has been made, that this function of the circulation behaves in a way capable of explaining the facts.

An effect on the tone of the heart appears to be, in the light of these considerations, the outstanding phenomenon. And by tone is meant the diastolic size of the heart, for this in a given case is indicative of the length of fiber, the length of fiber being, in turn, "synonymous with physiological condition or fitness of the muscle fibre" (Patterson, Piper and Starling (15)). This, as Bodo (16) remarks, was correlated by Starling and Visscher (17) with the oxygen consumption of the heart and the consumption of this "was determined by the initial length of its muscular fibres, i.e., by its diastolic volume." The heart's tone may be measured, therefore, in terms of its diastolic volume, "provided that the work performed by the heart is maintained constant during the whole of the experiment." Since, in the experiments of Cohn and Stewart the measurements were made during periods when the dogs were in so-called basal states, the work which their hearts performed may be regarded as constant, and the effects on diastolic size, as measures of the effect of the action of digitalis on tone.[5] In the current experiments the situation is different. The amount of work which the hearts did, and the rate of the contractions varied, as well as the output per beat, although the resistance against which they worked was uniform. These were the effects which took place with deterioration of the preparation and they may be regarded as measures of the decrease in its effectiveness, expressed ultimately as increase in its diastolic size. If change in diastolic size is expressive of change in tone, change in tone took place in these experiments as well as in the ones formerly reported (2*a*, *b*) (Cohn and Stewart).

---

[5] There is an obvious error in this statement; we have unfortunately been unable to trace its history. It is, of course, incorrect to correlate work with basal states.

CONCLUSIONS

1. The minute output from failing, dilated hearts in dogs in heart-lung preparations is increased when "therapeutic" doses of digitalis are administered. This effect is the opposite of that in the case of healthy hearts, normal in size.
2. When the output increases, the pressure in the right auricle decreases.
3. Increase in output is consistent with decrease in the diastolic volume of the heart.
4. If the inflow, and consequently the outflow, from the heart is restricted, the decrease in outflow is greater in the failing heart than in the same heart when it acts under the influence of digitalis. It appears from this test and from the discussion in the text that constriction of the hepatic veins is not a significant factor in the effect which the action of digitalis exerts on the size of the failing heart.
5. In estimating the value of a drug, its usefulness in therapeutics need not depend on its effect of any given function which presumably is correlated with the effect of that drug on the organism as a whole.

Addendum: After the completion of this report, the investigations of W. Bauer, H. H. Dale, L. T. Poulsson, and D. W. Richards, on "The control of the circulation through the liver" (J. Physiol., 1932, lxxiv, 343) appeared. A throttle mechanism in the livers of dogs was found to exist, but not one in cats and goats. Species obviously vary markedly in the possession of this function. Even when present, as in dogs, its influence depends on many factors, including constriction or dilatation of splanchnic vessels so that inferences in other connections, as for example its effect on the accumulation of blood in the abdominal vessels, apart from direct experiment, should not be drawn.

BIBLIOGRAPHY

1. Harrison, T. R., and Leonard, B. W., J. Clin. Invest., 1926, iii, 1. The Effect of Digitalis on the Cardiac Output of Dogs and Its Bearing on the Action of the Drug in Heart Disease.
2. (*a*) Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1928, vi, 53. The Relation between Cardiac Size and Cardiac Output per Minute Following the Administration of Digitalis in Normal Dogs.
   (*b*) Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1928, vi, 79. The Relation between Cardiac Size and Cardiac Output per Minute Following the Administration of Digitalis in Dogs in Which the Heart is Enlarged.
3. Dock, W., and Tainter, M. L., J. Clin. Invest., 1930, viii, 467. The Circulatory Changes after Full Therapeutic Doses of Digitalis with a Critical Discussion of Views on Cardiac Output.
4. Tainter, M. L., and Dock, W., J. Clin. Invest., 1930, viii, 485. Further Observations on the Circulatory Actions of Digitalis and Strophanthus with Special Reference to the Liver, and Comparisons with Histamine and Epinephrine.
5. Anitschkow, S. W., and Trendelenburg, P., Deutsche med. Wchnschr., 1928, liv, 1672. Die Wirkung des Strophanthins auf das suffiziente und auf das insuffiziente Warmblüterherz.
6. Bijlsma, U. G., and Roessingh, M. J., Arch. f. exper. Pathol. u. Pharm., 1922, xciv, 235. Die Dynamik des Säugetierherzens unter dem Einfluss von Stoffen der Digitalisgruppe.
7. Plant, O. H., J. Pharm. and Exper. Therap., 1913–14, v, 603. A Note on the Efficiency of the Knowlton-Starling Isolated Heart-Lung Preparation for Testing the Cardiac Action of Drugs.
8. Knowlton, F. P., and Starling, E. H., J. Physiol., 1912, xliv, 206. The Influence of Variations in Temperature and Blood-pressure on the Performance of the Isolated Mammalian Heart.
9. Bayliss, L. E., Müller, E. A., and Starling, E. H., J. Physiol., 1928, lxv, 33. The Action of Insulin and Sugar on the Respiratory Quotient and Metabolism of the Heart-Lung Preparation.
10. Lampe, W., and Méhes, J., Arch. f. exper. Pathol. u. Pharm., 1926, cxvii, 115. Gefässstudien an der überlebenden Warmblüterleber. II. Die Beeinflussung der Lebergefässe durch Strophanthin und Digitalisglykoside.
11. Grab, W., Janssen, S., and Rein, H., Ztschr. f. Biol., 1929, lxxxix, 324. Ueber die Grösse der Leberdurchblutung.
12. Popper, H., Klin. Wchnschr., 1931, x, 2129. Über Drosselvorrichtungen an Lebervenen.
13. Elias, H., and Feller, A., Stauungstypen bei Kreislaufstörungen mit besonderer Berücksichtigung der exudativen perikarditis. Julius Springer, Wien. u. Berlin, 1926.

14. Stewart, H. J., and Cohn, A. E., J. Clin. Invest., 1932, xi, 917. Studies on the Effect of the Action of Digitalis on the Output of Blood from the Heart. III. Part 1. The Effect on the Output in Normal Human Hearts. Part 2. The Effect on the Output of Hearts in Heart Failure with Congestion in Human Beings.
15. Patterson, S. W., Piper, H., and Starling, E. H., J. Physiol., 1914, xlviii, 465. The Regulation of the Heart Beat.
16. Bodo, R., J. Physiol., 1928, lxiv, 365. The Effect of the "Heart-tonics" and Other Drugs upon the Heart-tone and Coronary Circulation.
17. Starling, E. H., and Visscher, M. B., J. Physiol., 1927, lxii, 243. The Regulation of the Energy Output of the Heart.

[Reprinted from THE JOURNAL OF CLINICAL INVESTIGATION, September, 1932, Vol. 11, No. 5, pp. 897–916]

STUDIES ON THE EFFECT OF THE ACTION OF DIGITALIS ON THE OUTPUT OF BLOOD FROM THE HEART

II. THE EFFECT ON THE OUTPUT OF THE HEARTS OF DOGS SUBJECT TO ARTIFICIAL AURICULAR FIBRILLATION

BY HAROLD J. STEWART AND ALFRED E. COHN

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 16, 1932)

That an effect of giving digitalis to normal unanesthetized dogs is to decrease cardiac output and size, and to increase the extent of ventricular contractions has previously been reported (1). To study in dogs, phenomena comparable with heart failure with edema in human beings, experiments were undertaken employing several methods. The one finally adopted was to bring about, artificially, insufficiencies of the mitral valve (2). After a lesion had been made by operation, the hearts increased in size. The dogs exhibited no signs of heart failure, however, and were able to run on a treadmill as normal dogs do. When digitalis was given to them, the results were similar to those occurring in normal dogs. These were decrease in cardiac output and cardiac size, and increase in the extent of ventricular excursions (2). A situation was, however, still wanting, in which to test the effect of giving digitalis to animals the hearts of which were in a poor state functionally, a situation, in short, in which the output of blood per minute from the heart was diminished and the heart dilated. Stewart and Gilchrist (3) and Stewart and Crawford (4) have shown that such a situation could be managed. They found that when the hearts of normal dogs were made subject to auricular fibrillation, artificially induced, the volume output of blood per minute from the heart diminishes (3), and dilatation of the heart, detected by increase in size of its shadow in x-ray photographs, may occur (4). These changes, decrease in output and increase in size of the heart, were already present one hour after the onset of auricular fibrillation. Here then was a preparation suitable for testing in dogs the effect of giving digitalis when the functional capacity was diminished. In this report there are described, accordingly, observations on the effect of giving digitalis on the cardiac output per minute, cardiac size, ventricular and femoral pulse rates per minute in unanesthetized trained dogs during auricular fibrillation.

Methods

All the dogs were trained to breathe in a Benedict-Roth spirometer for 20 to 30 minutes a day for ten days to two weeks before they were used. At the end of this time they lay quietly on the table without emotional disturbance, while measurements of oxygen consumption were made and other procedures were carried out. It was possible to decide at the first or second trial whether a given dog was a satisfactory subject. In the technique of measuring oxygen consumption, the rubber mask described by Blalock was used (5).

The operative procedure has already been described (6). Briefly, wire electrodes were sutured to the right auricle (Figure 1). The operations were performed with sterile precautions, the dogs being anesthetized with ether given by the intratracheal method. After recovery from the preliminary operation, the auricles of the heart were made to fibrillate by stimulating them through these electrodes with faradic current obtained from one to three dry cell batteries placed in the primary circuit of a DuBois-Reymond induction coil. In order to be certain that the auricles were fibrillating, as well as to calculate the number of effective beats, a tracing of the femoral pulse was recorded simultaneously on the same film with an electrocardiogram (Figure 2) (7). The femoral pulse was transmitted by rubber tubing from a rubber cuff applied to the right hind-leg through an Erlanger capsule modified by Kolls (8), and Kubie (9), to a Frank capsule placed in front of the camera. The rubber cuff on the leg was inflated with air to a pressure near the diastolic level. The movements of a beam of light focused on a mirror glued to the membrane of the Frank capsule were reflected to the lens of the camera and were traced on the moving sensitive film simultaneously with the electrocardiogram. Nonpolarizable electrodes were placed on the right foreleg and the left hind-leg for the derivation of Lead II of the electrocardiogram.

Samples of arterial blood were drawn from a femoral artery and those of mixed venous blood from the right ventricle by a special cannula inserted into that chamber through the right external jugular vein (10). The oxygen contents of these samples were estimated by the Van Slyke and Neill manometric method (11) for calculation of the arteriovenous oxygen differences. Immediately after the blood samples were drawn, the oxygen consumption was measured with a Benedict-Roth spirometer equipped with a graphic recording drum. Data were available, therefore, for calculating the minute volume output of the heart according to the Fick principle (12).

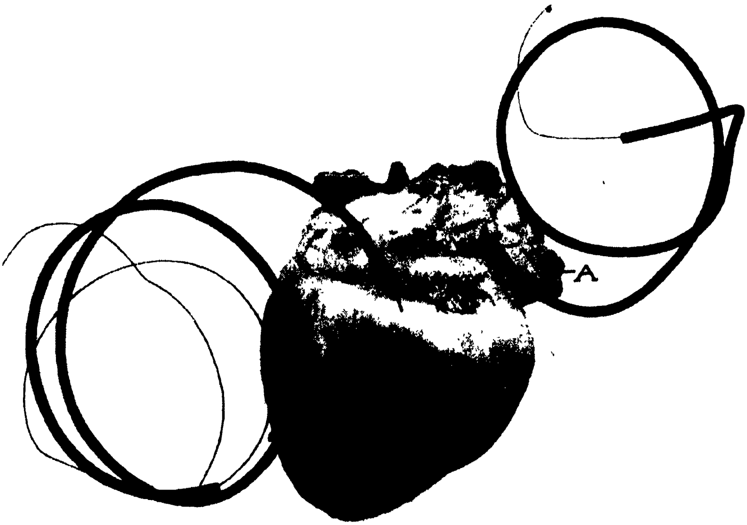
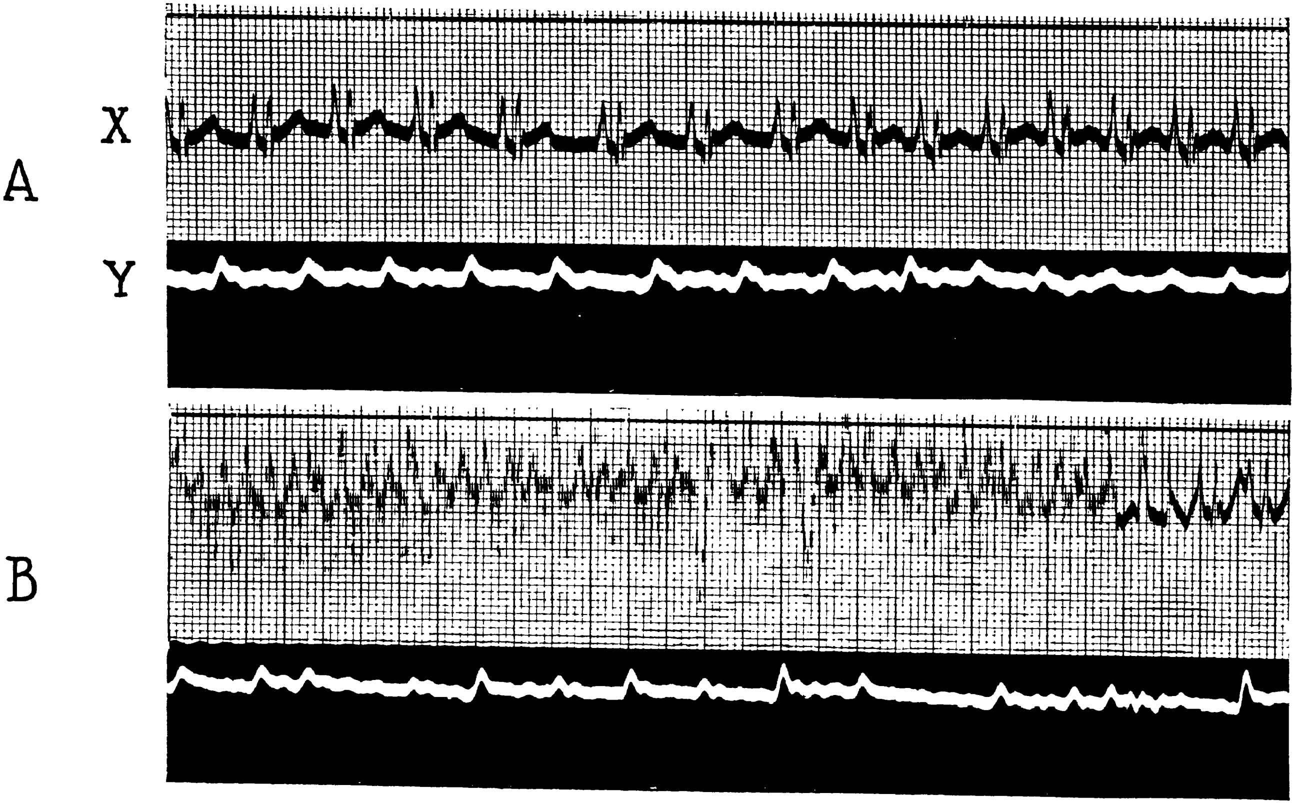


FIG. 1. This figure shows the manner in which the spiral wire electrodes were sutured to the right auricle.

A is the right auricular appendix.

X-ray photographs of the heart taken at a distance of 2 meters were made according to the method described by Stewart for obtaining photographs of the hearts of dogs under uniform conditions. The x-ray shadows of the heart were traced on thin paper and the areas measured with a planimeter (13).



X
A
Y
B

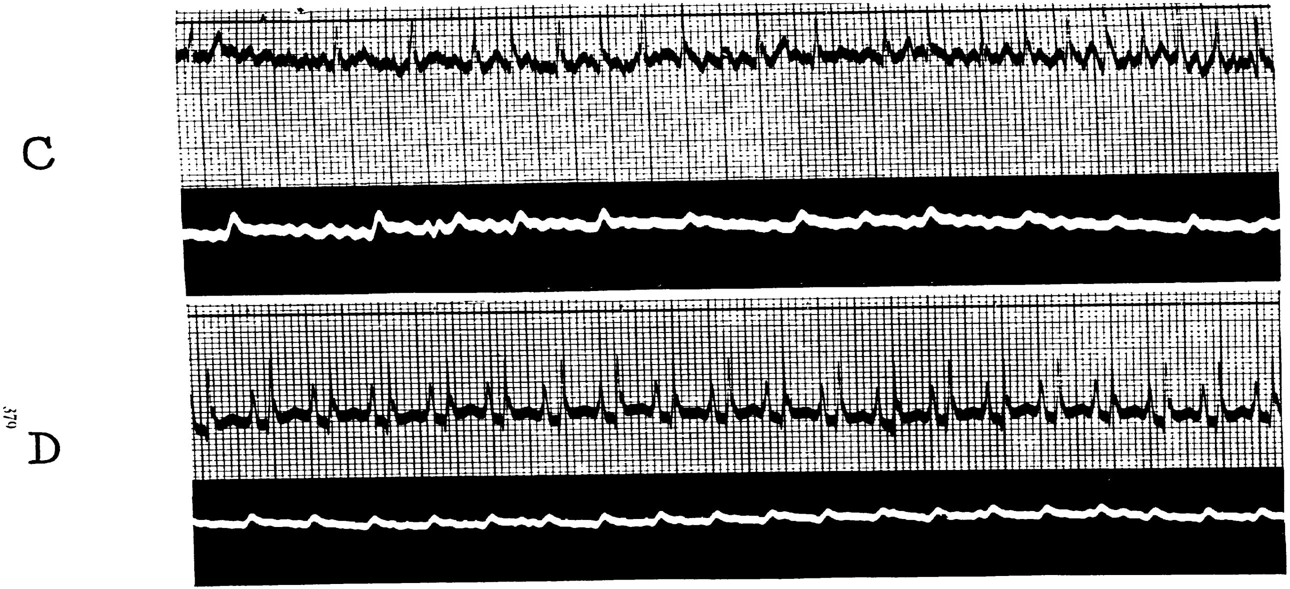


FIG. 2. In this figure are shown the records of dog 231, taken on April 14, 1927

A was taken when the rhythm was normal; *B*, during and after auricular fibrillation had been present 71 minutes; *C*, 200 minutes after the onset of auricular fibrillation and 105 minutes after the injection of tincture of digitalis (Upsher Smith) 2.0 cc. intravenously; and *D*, 81 minutes after the return to the normal rhythm, and 210 minutes after injecting digitalis. *X* is the electrocardiogram (Lead II) and *Y* the femoral pulse tracing. A short time interval elapses between the beginning of the QRS complex of the electrocardiogram and the corresponding femoral pulse, due partly to the time required for transmission of impulses from the heart to the femoral artery, and partly to that from the rubber cuff to the Frank capsule. In the femoral pulse tracing taken during auricular fibrillation (*B* and *C*) attention is called to the variation in the excursion of the femoral pulse from beat to beat. In this figure divisions of the ordinates equal 10^{-4} volts; divisions of the abscissae equal 0.04 of a second. The original curves are sharply contrasted black and white; no half tones are lost by the method of reproduction. The curves are reproduced full size.

TABLE 1

The Effect of Giving Digitalis on the Cardiac Output, Cardiac Size, and Ventricular Rate in Dogs Subject to Artificial Auricular Fibrillation

Dog number and sex	Date	Time with reference to faradic stimulation‖	Time with reference to administration of digitalis††	Weight	O₂ content		Arteriovenous oxygen difference	O₂ consumption	Cardiac output	Cardiac output per cent of initial	Cardiac area	Cardiac area per cent of initial	O₂ capacity	Ventricular rate*	Corresponding femoral pulse rate*	Digitalis	Rhythm of heart
					Arterial blood	Mixed venous blood											
	1927	*minutes*	*minutes*	*k.m.*	*volumes per cent*	*volume per cent*	*volumes per cent*	*cc. per minute*	*cc per minute*	*per cent*	*sq. cm.*	*per cent*	*volumes per cent*	*per minute*	*per minute*	*cc.*	
231 ♂	Apr. 14	0–65 B	B	10.45	25.24	17.88	7.36	82	1112	100	36.5	100	26.05	150	150		N.R.†
														150	150		
		60–75 A	B		25.49	15.77	9.72	84	864	78	39.6	109	26.05	330	120		A.F.¶
														300	130		
														330	180		
		180–220 A	85–120 A		24.62	15.30	9.32	91	976	88	36.0	99	24.95	190	130	2.0‡	A.F.
														200	200		
														120	110		
														150	150		
		64–89 E	190–230 A		23.77	16.71	7.06	89	1261	113	36.5	100	26.19	180	180		N.R.
														190	190		
														180	180		
	Apr. 21	0–45 B	B	9.15	15.79	9.63	6.16	95	1542	100	40.2	100	15.72	100	100		N.R.
														90	90		
		60–100 A	B		16.24	5.47	10.77	119	1105	72	42.5	106	16.53	270	170		A.F.
														290	150		
		175–205 A	90–95 A		15.57	5.38	10.19	125	1227	80	39.5	98	15.95	180	170	4.66§	A.F.
		60–90 E	180–185 A		15.46	6.52	8.94	104	1163	75	37.6	94	16.02	170	170		N.R.
														170	170		

TABLE 1—*Continued*

Dog number and sex	Date	Time with reference to faradic stimulation‖	Time with reference to administration of digitalis††	Weight	O_2 content		Arteriovenous oxygen difference	O_2 consumption	Cardiac output	Cardiac output per cent of initial	Cardiac area	Cardiac area per cent of initial	O_2 capacity	Ventricular rate*	Corresponding femoral pulse rate*	Digitalis	Rhythm of heart
					Arterial blood	Mixed venous blood											
	1927	*minutes*	*minutes*	*kgm.*	*volumes per cent*	*volumes per cent*	*volumes per cent*	*cc. per minute*	*cc. per minute*	*per cent*	*sq. cm.*	*per cent*	*volumes per cent*	*per minute*	*per minute*	*cc.*	
229 ♀	Mar. 25	0–75 B	B	11.8	20.81	12.34	8.47	145	1716	100	48.1	100	21.28	150	150		N.R.
														150	150		
		60–85 A	B		20.31	8.80	11.51	141	1220	71	50.0	104	21.26	340	140		A.F.
														340	130		
														310	180		
														360	180		
																2.5‡	
		160–210 A	55–105 A		19.80	10.53	9.27	144	1554	90	46.7	97	21.64	240	200		A.F.
														240	240		
		65–110 E	170–215 A		19.58	11.38	8.27	123	1487	86	45.4	95	20.68	190	190		N.R.
														190	190		
192 ♂	Mar. 29	0–35 B	B	19.1	23.63	15.93	7.70	220	2853	100	59.3	100	24.46	200	200		N.R.
														200	200		
		70–105 A	B		23.29	13.55	9.74	220	2258	79	65.8	111	23.86	300	170		A.F.
														360	240		
														310	140		
														330	150		
														330	120		
														300	150		
														300	180		
																4.0‡	
		180–225 A	60–110 A		21.35	12.32	9.03	223	2469	87	63.3	107	23.16	240	230		A.F.
														240	240		
		57–98 E	172–219 A		22.74	13.06	9.68	190	1963	69	58.5	98	22.65	190	190		N.R.
														190	190		
		60–85 A**	150–185 A		22.24	12.52	9.72	223	2294	80	61.1	103	23.83	200	200		A.F.

TABLE 1—*Concluded*

Dog number and sex	Date	Time with reference to faradic stimulation‖	Time with reference to administration of digitalis††	Weight	O_2 content		Arteriovenous oxygen difference	O_2 consumption	Cardiac output	Cardiac output per cent of initial	Cardiac area	Cardiac area per cent of initial	O_2 capacity	Ventricular rate*	Corresponding femoral pulse rate*	Digitalis	Rhythm of heart
					Arterial blood	Mixed venous blood											
	1927	*minutes*	*minutes*	*kgm.*	*volumes per cent*	*volumes per cent*	*volumes per cent*	*cc. per minute*	*cc. per minute*	*per cent*	*sq. cm.*	*per cent*	*volumes per cent*	*per minute*	*per minute*	*cc.*	
234 ♀	Apr. 28	0–49 B	B	15.2	24.86	14.07	9.99	113	1131	100	50.0	100	25.69	200	200		N.R.
														190	190		
		60–80 A	B		25.49	13.73	11.76	104	884	78	53.0	106	26.32	300	180		A.F.
														330	150		
																8.0§	
		140–170 A	45–75 A		24.86	17.96	6.88	108	1569	138	48.0	96	24.32	220	220		A.F.
		60–95 E	153–185 A		24.03	16.44	7.59	112	1449	127	46.3	93	25.98	190	190		N.R.
														190	190		

‖ B = before beginning stimulation, A = after beginning stimulation, E = after ending stimulation; see text "Plan of Observations."

†† B = before injecting digitalis, A = after injecting digitalis.

* In this table as well as in Table 2, the ventricular rate and the corresponding femoral pulse rate were counted in the electrocardiographic record and simultaneous femoral pulse tracing.

† N.R. = normal rhythm.

¶ A.F. = auricular fibrillation.

‡ Tincture of digitalis (Upsher Smith) given intravenously.

§ Digifoline (Ciba) given intravenously.

** In this dog observations were made during a second "period of auricular fibrillation with digitalis."

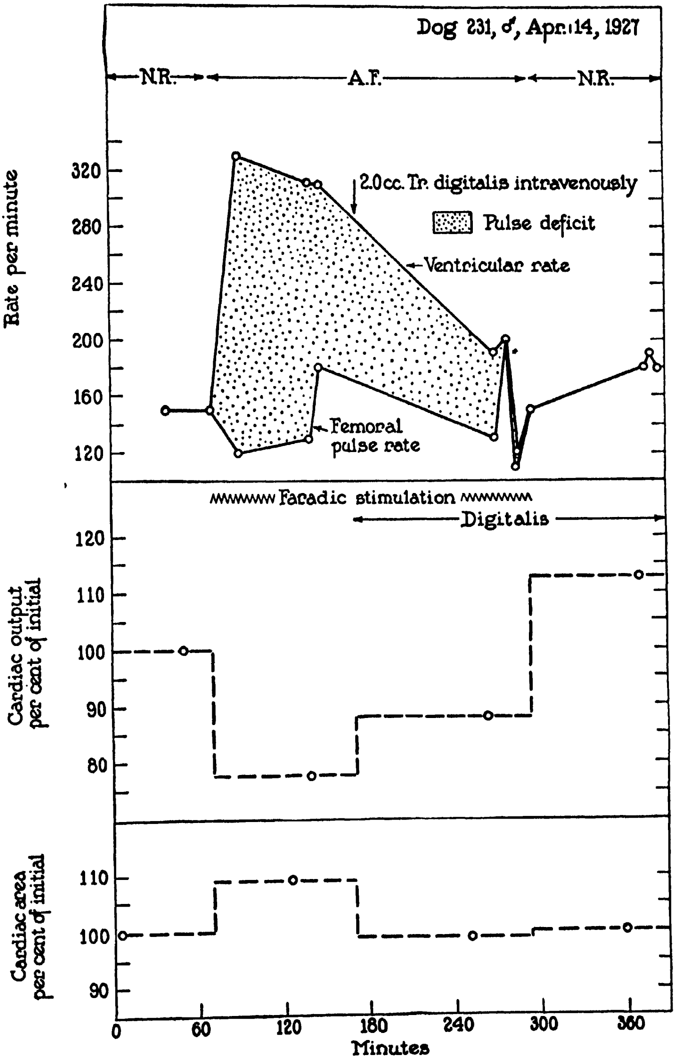


FIG. 3. In this figure is shown the effect of giving digitalis on cardiac output, cardiac size, ventricular rate, and femoral pulse rate per minute in Dog 231 (April 14, 1927) during artificial auricular fibrillation.

In this figure as well as in Figure 4, the points on the curves show the exact time (abscissa) at which each observation was made. In order to bring into relief the relations between the various situations at these times, the curves are drawn in dotted straight lines as though these relations existed throughout the various periods. In this figure and in Figure 4, N.R. indicates normal rhythm and A.F., auricular fibrillation.

Plan of Observations

The day after the preliminary operation the following observations were made. The dogs, in a basal metabolic state, lay quietly on the table without anesthesia. A set of observations consisting of: an x-ray photograph of the heart, samples of arterial and of mixed venous blood, measurement of oxygen consumption, and electrocardiograms together with simultaneous femoral pulse tracings, was taken during the period of normal cardiac rhythm, hereafter called the "period of normal rhythm." Taking a complete set of observations required usually 30 to 45 minutes. The right auricle was then made subject to fibrillation. At the end of approximately one hour, designated the "period of auricular fibrillation," while the auricles were still fibrillating, a second set of observations was made. Then, while this rhythm was maintained by continuing faradic stimulation, tincture of digitalis (Upsher Smith) was injected intravenously in an amount calculated to be 25 to 30 per cent of the lethal dose.[1] Approximately one hour later, during the "period of auricular fibrillation with digitalis," the auricles still fibrillating, a third set of observations was made. Stimulation of the auricles was then discontinued and the rhythm of the heart became normal again. After another hour, designated the "period of normal rhythm with digitalis," a fourth set of observations was made. Deviation from this schedule, whenever it occurred, is shown in the tables.

There are complete observations in four dogs. The results secured in the case of dog 231 serve to illustrate the course of events (Table 1, Figure 3). When the heart was beating normally at a rate of 150 per minute its output was 1112 cc. per minute, and the area of the heart measured 36.5 sq. cm. (Table 1, April 14, 1927). One hour after the onset of auricular fibrillation and while this rhythm was still present, the ventricular rate being 300 to 330 (Figure 2) and the femoral pulse rate 120 to 180 per minute, the cardiac output fell to 864 cc. per minute or 78 per cent of its initial value (Table 1, Figure 3), and the area of the heart measured 39.6 sq. cm., an increase to 109 per cent of its initial size. While the auricles were fibrillating, tincture of digitalis (Upsher Smith) 2.0 grams was given intravenously. Eighty-five minutes later, under the influence of digitalis, the ventricular rate decreased further from 200 to 120, and the corresponding femoral pulse rate from 200 to 110 per minute, the pulse deficit being smaller; the heart was, in short, still beating irregularly but more slowly and more forcibly, there being only a few ineffective beats. The cardiac output *increased* to 976 cc. per minute (88 per cent of its initial value) and its size *decreased* to 36.0 sq. cm. (99 per cent of its initial size). Then stimulation of the auricles was discontinued and the rhythm of the heart again became normal. One hour later the cardiac rate was 190 to 200 per minute, the heart still being under the influence of digitalis. Since there was no longer a pulse deficit, the cardiac output increased to 1261 cc. per minute (113 per cent of the initial) though the area of the heart remained unchanged (36.5 sq. cm., 100 per cent). When the observations were repeated one week later (April 21, 1927) similar results were obtained (Table 1), with this exception that cardiac output and cardiac size both diminished when the rhythm of the heart became regular again in the final (fourth) period.

---

[1] This amount was chosen because Robinson and Wilson (14) found the therapeutic dose of digitalis for cats to be 30 per cent of the calculated lethal dose, and because Cohn (15) found that the dose for cats must be multiplied by the factor 1.16 to arrive at a comparable quantity for dogs. We have accordingly injected this amount of the tincture. To several dogs we administered digifoline (Ciba) 0.5 cc. per kilogram of body weight (16, 17). The same phenomena resulted irrespective of the preparation that was given.

Summary: With the onset of auricular fibrillation the cardiac output decreased and the heart dilated. When digitalis was given the size diminished and the output increased; that is to say, the capacity of the heart to expel blood increased. The results in the three other dogs were similar to these. The most frequent effect observed after the end of stimulation, when the rhythm again became regular, was further decrease in the size of the heart and a decrease in output. In other instances, as in the one described in detail, cardiac size remained unchanged from that in the fibrillatory state and the cardiac output increased.

In four untrained dogs similar observations were made (Table 2). The arteriovenous oxygen difference was measured representing, as it does, the oxygen consumed by the tissues per liter of blood, and from

TABLE 2

The Effect of Giving Digitalis on the Relative Blood Flow, Cardiac Size, and Ventricular Rate in Dogs Subject to Artificial Auricular Fibrillation

Dog number and sex	Date	Time with reference to faradic stimulation‖	Time with reference to administration of digitalis††	Weight	O_2 content		Arteriovenous oxygen difference*	Blood flow per cent of initial	Cardiac area	Cardiac area per cent of initial	O_2 capacity	Ventricular rate	Corresponding femoral pulse rate	Digitalis†	Rhythm of heart
					Arterial blood	Mixed venous blood									
		minutes	*minutes*	*kgm.*	*mM.*	*mM.*	*mM.*	*per cent*	*sq. cm.*	*per cent*	*mM.*	*per minute*	*per minute*	*cc.*	
226 ♀	February 11, 1927	0–13 B	B	14.75	11.25	8.17	2.99	100	45.2	100	11.81	200	200		N.R.¶
												190	190		
												190	190		
		70–105 A	B		11.80	6.72	5.08	59	50.7	112	12.04	350	160		A.F.§
												340	160		
												350	150		
		170–189 A	60–79 A		11.07	7.70	3.37	89	47.0	104	12.22	270	270	2.8	A.F.
												210	210		
		60–80 E	150–170 A		11.11	7.08	4.03	74	42.8	95	12.06	190	190		N.R.
												200	200		
												190	190		
214 ♀	December 16, 1926	0–50 B	B	13.88	10.47	7.14	3.33	100	44.4	100	9.84	230	230		N.R.
		73–143 A	B		10.56	5.29	5.27	63	50.4	113	10.75	340	190		A.F.
		208–268 A	60–120 A		10.52	6.91	3.61	92	44.8	101	10.36	200	200	3.0	A.F.
		60–68 E	202–210 A		10.89	6.30	4.59	72	41.6	94	11.15	210	210		N.R.

TABLE 2—*Concluded*

Dog number and sex	Date	Time with reference to faradic stimulation‖	Time with reference to administration of digitalis††	Weight	O_2 content		Arteriovenous oxygen difference*	Blood flow per cent of initial	Cardiac area	Cardiac area per cent of initial	O_2 capacity	Ventricular rate	Corresponding femoral pulse rate	Digitalis†	Rhythm of heart
					Arterial blood	Mixed venous blood									
		minutes	*minutes*	*kgm.*	*mM.*	*mM.*	*mM.*	*per cent*	*sq. cm.*	*per cent*	*mM.*	*per minute*	*per minute*	*cc.*	
220 ♀	January 19, 1927	0–22 B	B	10.2	11.10	8.36	2.74	100	34.3	100	11.21	130	130		N.R.
												140	140		
		60–90 A	B		11.21	6.84	4.37	62	37.8	110	11.40	300	210		A.F.
												350	240		
												340	210		
														2.3	
		180–210 A	60–90 A		10.29	6.53	3.76	73	34.6	100	10.40	160	150		A.F.
		210–270 A‡	135 A		10.74	6.92	3.82	72			11.45	200?	190		A.F.
		75–100 E	360–385 A		11.08	6.63	4.45	61	31.3	91	11.39	180	180		N.R.
												190	190		
223 ♀	February 3, 1927	0–40 B	B	11.0	11.09	8.84	2.25	100	39.2	100	11.88	160	160		N.R.
		40–60 A	B		11.70	6.77	4.93	46	42.5	109	11.90	340	180		A.F.
														2.0	
		115–150 A	47–82 A		11.04	6.96	4.08	55	41.9	106	12.39	180	180		A.F.
		60–80 E	137–157 A		11.47	6.50	3.97	57	40.9	104	12.17	170	170		N.R.

‖ B = before beginning stimulation, A = after beginning stimulation, E = after ending stimulation; see text "Plan of Observations."

†† B = before injecting digitalis, A = after injecting digitalis.

* Oxygen removed from each liter of blood.

† Tincture of digitalis (Upsher Smith) given intravenously.

¶ N.R. = normal rhythm.

§ A.F. = auricular fibrillation.

‡ In this dog observations were made during a second "period of auricular fibrillation with digitalis."

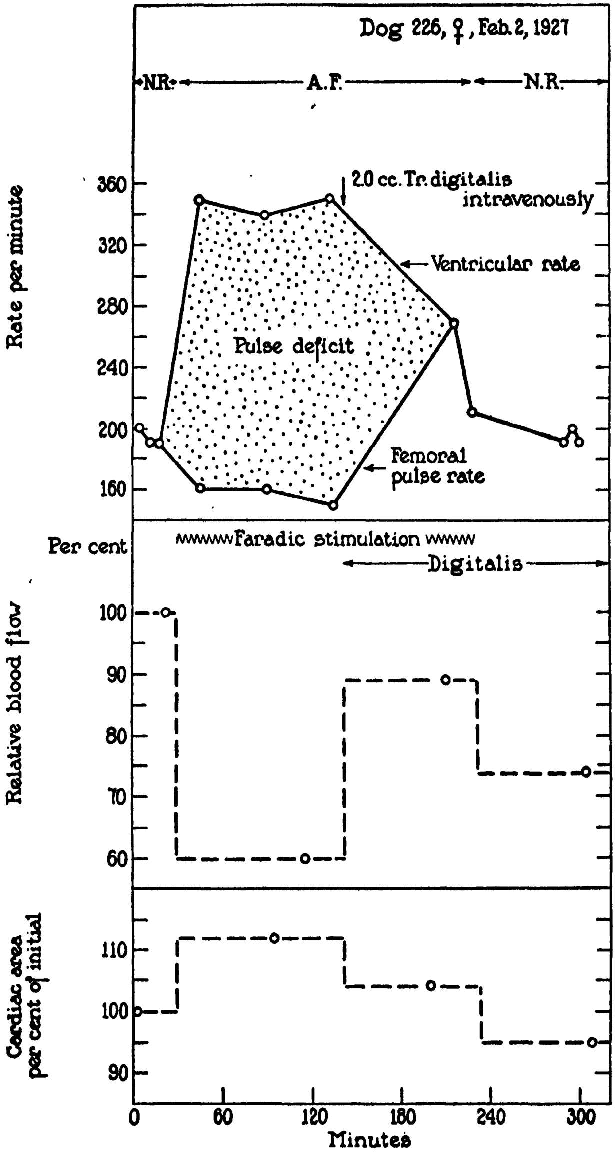


FIG. 4. In this figure is shown the effect of giving digitalis on the relative blood flow, cardiac size, ventricular and femoral pulse rates per minute in dog 226 during artificial auricular fibrillation.

this the relative blood flow[2] (6) has been calculated, the control period being placed at 100; the results in all these measurements are similar to those in which the cardiac output was measured. Dog 226 (Table 2, Figure 4) may serve to illustrate the course of events. There was decrease in relative blood flow and increase in cardiac size when the cardiac mechanism was that of auricular fibrillation; increase in output and decrease in size when digitalis was given during this rhythm and finally, decrease in output and decrease in cardiac size with the return of the normal rhythm. The results in the three other dogs are similar.

A Note on the Effect of Giving Digitalis to a Dog in the Presence of Edema Artificially Induced

In dog 265, edema of the extremities occurred as a result of taking sodium bromide[3] (18, 19, 20, 21). The fluid intake was uniform, being 1500 cc. a day. The diet consisted of milk and eggs given by stomach tube at the same time each evening. The output of urine was measured. The dog was trained in the use of the spirometer for measuring oxygen consumption. All measurements were made when the dog was in a basal metabolic state. On November 27, 1928, when edema appeared, the cardiac output measured 1550 cc. per minute and the area of the heart 48.3 sq. cm.; the left and right ventricular excursions measured in moving x-ray films (1) 2.8 mm. and 3.4 mm. respectively (Table 3, Figure 5). Tincture of digitalis (Upsher Smith) 2.7 cc. was given intravenously to this dog after these measurements had been made. The output of urine increased, the dog lost weight, and became free of edema on November 30. Twenty-four hours after the administration of digitalis, cardiac output *increased* to 2100 cc.

---

[2] "Relative blood flow" is a ratio between some state of flow and another, for example, between the arteriovenous oxygen difference per liter of blood in a normal cardiac mechanism and an abnormal one, the metabolism, that is to say, the oxygen consumption, remaining constant.

[3] This dog was given by stomach tube sodium chloride 15.0 grams (10 per cent solution) a day for 5 days. She was then given, in the same manner, sodium bromide 15.0 grams (10 per cent solution) a day for 5 days, when she became drowsy. Four days later (November 27, 1928) edema of the extremities was observed. Water was given in the morning with sodium chloride or sodium bromide, and milk and eggs in the evening to bring the total daily intake of fluid up to 1500 cc. The caloric value of the food intake was constant.

TABLE 3

The Effect of Giving Digitalis on the Cardiac Output, Cardiac Size, and Extent of Ventricular Excursions in Dog 265 (♀) Subject to Edema after Taking Sodium Bromide

Date	Time with reference to administration of digitalis	Weight	O_2 content		Arteriovenous oxygen difference	O_2 consumption	Cardiac output	Cardiac output per cent of initial	Cardiac area*	Cardiac area per cent of initial	O_2 capacity	Ventricular excursions**		Presence of edema	Digitalis***
			Arterial blood	Mixed venous blood								Left	Right		
1928		*kgm.*	*volumes per cent*	*volumes per cent*	*volumes per cent*	*cc. per minute*	*cc. per minute*	*per cent*	*sq. cm.*	*per cent*	*per cent*	*mm.*	*mm.*		*cc.*
November 27	Before	14.0	18.86	11.47	7.39	115	1550	100	48.3	100	21.09	2.8	3.4	Yes	2.7
November 28	24 hours after	13.4	15.45	10.50	4.95	105	2100	136	47.5	98	19.03	4.8	4.8	Yes	
November 30	72 hours after	12.8	15.85	11.57	4.28	110	2550	173	42.4	88	18.41	5.2	5.6	No	

* These x-ray photographs were taken at a distance of 34 inches.

** Measured from tracings made of the x-ray moving films (Cohn and Stewart (1)).

*** Tincture of digitalis (Upsher Smith) given intravenously.

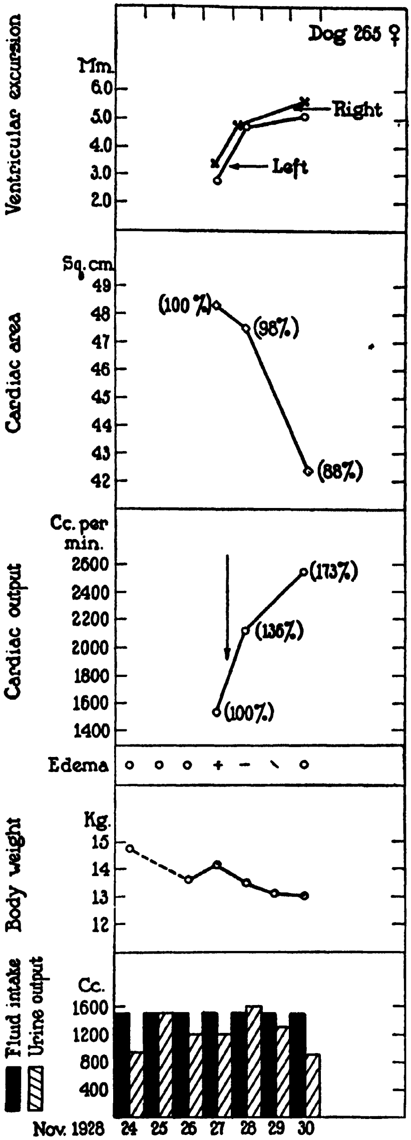


FIG. 5. In this figure is shown the effect of giving digitalis on cardiac output, cardiac size, and extent of ventricular excursions in dog 265, subject to edema, the result of oral ingestion of sodium bromide.

In interpreting the symbols relating to the presence of edema, o = absent, + = present, — = unchanged, and ╲ = decreasing.

(136 per cent of the initial output), cardiac size decreased to 47.5 sq. cm. (89 per cent), and the left and right ventricular excursions both increased (4.8 mm. and 4.8 mm. respectively). Two days later still, when edema had quite disappeared, the cardiac output *increased* still further to 2550 cc. (173 per cent), cardiac size diminished further to 42.4 sq. cm. (88.0 per cent), and the ventricular excursions increased to 5.2 mm. (left) and 5.6 mm. (right).

Summary: When digitalis was given to a dog, the subject of edema artifically induced, cardiac output increased, cardiac size diminished, and the extent of ventricular excursions increased. The dog lost weight and became free of edema.

DISCUSSION

That the effect of giving digitalis to intact normal dogs is to decrease cardiac output and cardiac size and to increase ventricular excursions appears to be a fact (1, 17). Its effect in dogs in which the hearts were enlarged, but in which there was no evidence of heart failure has also been studied. In this situation, as in the normal one, the effect was to decrease cardiac output and cardiac size and to increase the extent of ventricular excursions (2). The effect of giving digitalis has been studied therefore under the following circumstances. First, in normal dogs the hearts of which were normal in size; second, in dogs the subject of muscular hypertrophy following the creation of artificial valvular lesions. In both these, the result is decrease in output. And now there is a case in which information was still lacking, namely, in intact, unanesthetized dogs in which the cardiac output is diminished and the heart dilated due to the action of auricular fibrillation artificially induced. The result is *increase* in cardiac output and *decrease* in cardiac size. Insufficient hearts become smaller, it seems, and, unlike normal hearts in which it decreases, increase their output as the result of a change dependent on the heart itself, and obviously independent of changes in a peripheral mechanism, such as that of the throttle action of the hepatic veins. The outstanding point is this, that at a time when the basic mechanism of the heart beat remained unchanged, the action of digitalis resulted in increase in output, though the rate decreased; occasionally it increased the output still more when the normal rhythm was restored, though the normal did not attain the fibrillatory rate.

In these observations and in the ones published in the third paper (22) parallel phenomena have been studied in dogs and in human beings, namely, the effect of giving digitalis to normal animals and to normal human beings on the one hand; on the other, the effect in dogs in which the cardiac output is diminished and dilatation is present, and in patients the subjects of heart failure, whose hearts are enlarged and their output diminished. The output, both in normal dogs and in normal men, as well as the size, decrease; in diseased human beings and in abnormal dogs during heart failure, increase in output and decrease in size take place. Discussion of these results will be found in a third report (22). It is sufficient to state now that digitalis seems to exert the same essential effects both in normal and diseased hearts; it decreases cardiac size, its effect on tone, and it increases the extent of ventricular contraction, its effect on contraction (1, 2).

Edema which results in dogs from the oral administration of large doses of sodium bromide is probably not analogous to edema occurring in heart failure. The mechanism of its occurrence is not clearly known. It is presumably the result of shifting of ions in the blood, so that bromide ions are substituted for chloride ions; in consequence of this process, water is retained in the tissues. The course of events following the administration of digitalis in this condition resembles that in heart failure in human beings, namely, the cardiac output increases, the cardiac size decreases, and the extent of ventricular excursion increases.

SUMMARY AND CONCLUSIONS

1. During auricular fibrillation when the ventricular rate is rapid the cardiac output per minute is less than it is during the normal, slower sinus rhythm (3). In consequence of this abnormal rhythm in intact unanesthetized trained dogs the heart increases in size. This conclusion is based on a larger number of observations than was possible in an earlier paper (4).
2. When the cardiac output is diminished and the heart is dilated due to artificial auricular fibrillation, the administration of digitalis results in increase in cardiac output and decrease in cardiac size.
3. When the normal rhythm returns, the heart being, of course, still under the influence of digitalis, the output either increases, the size

remaining unchanged (from that in the fibrillatory state), or both output and size decrease.

4. The observations show, as do the ones next to be reported, that digitalis has the same action in normal and in pathological hearts; it decreases cardiac size (an effect on tone). The amount of the cardiac output which results from this action depends upon the initial size of the heart; it decreases in normal hearts, and increases in dilated ones.

5. In a dog, the subject of edema due to taking sodium bromide, the administration of digitalis increased cardiac output, decreased cardiac size, and increased the extent of ventricular excursions.

BIBLIOGRAPHY

1. Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1928, vi, 53. The Relation between Cardiac Size and Cardiac Output per Minute Following the Administration of Digitalis in Normal Dogs.
2. Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1928, vi, 79. The Relation between Cardiac Size and Cardiac Output per Minute Following the Administration of Digitalis in Dogs in Which the Heart is Enlarged.
3. Stewart, H. J., and Gilchrist, A. R., J. Clin. Invest., 1928, v, 335. Studies of the Effect of Cardiac Irregularity on the Circulation. II. The Estimation of Cardiac Output in Dogs Subject to Artificial Auricular Fibrillation.
4. Stewart, H. J., and Crawford, J. H., J. Clin. Invest., 1927, iii, 483. The Effect of Regular and Irregular Tachycardias on the Size of the Heart.
5. Blalock, A., J. Lab. and Clin. Med., 1927, xii, 378. A Rubber Mask for Determination of Oxygen Consumption of the Dog.
6. Stewart, H. J., Crawford, J. H., and Hastings, A. B., J. Clin. Invest., 1926, iii, 435. The Effect of Tachycardia on the Blood Flow in Dogs. I. The Effect of Rapid Irregular Rhythms as Seen in Auricular Fibrillation.
7. Stewart, H. J., Crawford, J. H., and Gilchrist, A. R., J. Clin. Invest., 1928, v, 317. Studies on the Effect of Cardiac Irregularity on the Circulation. I. The Relation of Pulse Deficit to Rate of Blood Flow in Dogs Subject to Artificial Auricular Fibrillation and to Regular Tachycardia.
8. Kolls, A. C., J. Pharm. and Exper. Therap., 1920, xv, 443. An Indirect Method for the Determination of Blood Pressure in the Unanesthetized Dog.
9. Kubie, L. S., J. Lab. and Clin. Med., 1925–26, xi, 186. A Substitute for the Rubber Bulb for Use in the Kolls and Erlanger Blood Pressure Instruments.
10. Stewart, H. J., J. Biol. Chem., 1925, lxii, 641. The Oxygen and Carbon Dioxide Contents of the Arterial and Mixed Venous Blood in Normal Intact Dogs.

11. Van Slyke, D. D., and Neill, J. M., J. Biol. Chem., 1924, lxi, 523. The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement. I.
12. Fick, A., Verhandl. d. Würzb. physikal.-med. Gesellsch., 1870, N. F. ii. S. xvi. der Sitzungsber. Ueber die Messung des Blutquantums in der Herzventrikeln.
13. Stewart, H. J., J. Clin. Invest., 1926, iii, 475. A Technique for Measuring X-ray Photographs of the Cardiac Areas of Dogs.
14. Robinson, G. C., and Wilson, F. N., J. Pharm. and Exper. Therap., 1918, x, 491. A Quantitative Study of the Effect of Digitalis on the Heart of the Cat.
15. Cohn, A. E. Unpublished experiments.
16. Pardee, H. E. B., J. Am. Med. Assoc., 1925, lxxxv, 1359. Hypodermic Digitalis Preparations.
17. Harrison, T. R., and Leonard, B. W., J. Clin. Invest., 1926, iii, 1. The Effect of Digitalis on the Cardiac Output of Dogs and Its Bearing on the Action of the Drug in Heart Disease.
18. Hastings, A. B., and van Dyke, H. B., J. Biol. Chem., 1928, lxxviii, p. xxxv. Studies of the Bromide and Chloride Distribution in the Blood of Dogs and the Production of Experimental Edema by Sodium Bromide Administration.
19. Hastings, A. B., and van Dyke, H. B., J. Biol. Chem., 1931, xcii, 13. Studies of Bromide Distribution in the Blood. I. In Vitro Experiments of Bromide and Chloride Distributions.
20. Hastings, A. B., and van Dyke, H. B., J. Biol. Chem., 1931, xcii, 27. Studies of Bromide Distribution in the Blood. II. The Distribution of Bromides and Chlorides in the Blood of Dogs Following the Oral Administration of Sodium Bromide.
21. Hastings, A. B., Harkins, H. N., and Liu, S. K., J. Biol. Chem., 1932, xciv, 681. Blood and Urine Studies Following Bromide Injection.
22. Stewart, H. J., and Cohn, A. E., J. Clin. Invest., 1932, xi, 917. Studies on the Effect of the Action of Digitalis on the Output of Blood from the Heart. III. Part 1. The Effect on the Output in Normal Human Hearts. Part 2. The Effect on the Output of Hearts in Heart Failure with Congestion, in Human Beings.

[Reprinted from The Journal of Clinical Investigation, September, 1932, Vol. 11, No. 5, pp. 917–955]

STUDIES ON THE EFFECT OF THE ACTION OF DIGITALIS ON THE OUTPUT OF BLOOD FROM THE HEART. III[1,2]

Part 1. The Effect on the Output in Normal Human Hearts

Part 2. The Effect on the Output of Hearts in Heart Failure with Congestion, in Human Beings

By HAROLD J. STEWART and ALFRED E. COHN

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, May 31, 1932)

PART I

The Effect on the Output in Normal Human Hearts

The action of digitalis when given to human beings is still incompletely understood. Investigations, in recent years (1, 2, 3, 4), which have been directed toward elucidating its influence on the behavior of the heart have led to a certain degree of confusion. The analysis of the results which have been obtained shows that inferences which were drawn were in part inexact, due to utilizing the results obtained in one situation, in the normal hearts of dogs for example or in those of human beings, in an explanation of the condition of disease. But beside difficulties in interpretation of this sort, error has also resulted from the use of insufficiently tested methods. This defect in the case of human beings applies, it seems, to the use of the method of Field, Bock, Gildea, and Lathrop (5) by certain investigators (1, 6), and to that of Henderson and Haggard (7) by others (8). The opportunity is still open, therefore, to study the minute volume output[3] of human

---

[1] A preliminary report of these observations appeared in the Proc. Soc. Exp. Biol. and Med., 1931, xxix, 207 and 209.

[2] An abstract of these studies was read before the American Society for Clinical Investigation, May 2, 1932.

[3] It is always the volume output of blood per minute to which reference is made when the word "output" is used.

hearts under the influence of digitalis when they are normal and also when they are the subjects of heart disease. This study is the more important since the result, observed in analyzing the effect on normal hearts, differs, as it turns out, so profoundly from that which supposedly obtains in enlarged diseased ones. If the two are really the same, fundamental conceptions concerning the circulation in heart failure require radical revision.

In this study digitalis was given to six normal individuals on seven occasions. Observations were made of its effect on cardiac output, on cardiac size, on arterial blood pressure, on venous blood pressure, on vital capacity, on cardiac rate, and on the electrocardiogram.

Measurements of cardiac output were made by the acetylene method of Grollman (9) which, so far as can now be judged, gives reasonably reliable results, for the cardiac output in man measured by it agrees with the amount estimated simultaneously by a direct method (10, 11). Our results, at all events, agree with those reported by Grollman (12).[4] Estimations of oxygen consumption for use in calculating the cardiac output were made with a Benedict-Roth spirometer. The diastolic size of the heart was traced from x-ray photographs taken at a distance of 2 meters, the subject being placed in a standing position, on thin paper and its area measured with a planimeter as suggested by Levy (13). Estimations of blood pressure were made by the auscultatory method. The three standard leads of the electrocardiogram were taken. Measurements of venous pressure were made by direct methods which will be described later. The vital capacity was measured in a spirometer.

Plan of Observations

After eating a moderate supper the subjects went to bed in the hospital the night before the observations began, and remained there at rest until 24 hours after the administration of digitalis. To assure their being in a basal metabolic state they were given small carbohydrate meals. The volume output was measured at 2, 4, 9, 12, and 15 hours after giving digitalis. The meals were taken just after the 4 and 15 hour measurements. Since from Grollman's observations three hours are required to return to a basal minute volume output (14), though the basal metabolic rate may still be elevated 1 or 2 per cent (15), this distribution made it certain that the measurements were proper. No water was given for three hours before each observation in order to avoid the effect of ingestion of fluid (16). The Gatch frame of the bed was raised one half-hour before observations so that subjects reclined at an angle of 135 degrees because Grollman found that mixing the gases during rebreathing takes place more satisfactorily in this position. Patients suffering from heart failure, moreover, often find it difficult or impossible to lie flat. We wished the two sets of investigations to be comparable from this point of view.

---

[4] We wish to express our thanks to Dr. E. K. Marshall for his kindness in placing at the disposal of one of us (H. J. S.) the facilities of his laboratory to learn this method, and to Dr. Arthur Grollman for his kindness in teaching him his method and discussing our data with him.

Counts of the pulse rate were made at intervals of a few minutes. At the end of a half-hour, subjects rebreathed from a bag containing a mixture of acetylene and air. Two proper samples of gas were taken for analysis and for calculation of the arteriovenous oxygen difference. The oxygen consumption was then estimated. Electrocardiograms were taken, followed by measurement of arterial and venous pressures and of vital capacity. Subjects were then wheeled in a chair to the roentgen laboratory to secure x-ray photographs of the heart. After being weighed they returned to bed to await taking the next observations. Records were made of the body temperature (rectal). The temperature of the room in which the studies were made was kept approximately constant (17). Analysis of the samples of gas for concentrations of carbon dioxide, oxygen, and acetylene was made in a modified Haldane apparatus (9).

Observations were made immediately before the administration of digitalis, at the intervals stated during the first 24 and at 24 hour intervals until the return of the initial cardiac output. The subjects had been made familiar with the procedures and were not disturbed by them. After the first 24 hours they attended to their usual daily routines except on days of observation when they remained in bed to assure their being in a basal metabolic state. The initial cardiac output in all except one was normal (Subject 4) and agreed with previous measurements in each case.

Observations

Digitalis in the form of digitan (Merck) 0.8 to 1.0 gram was given by mouth in a single dose. No one experienced nausea or vomiting.

Effect on cardiac minute volume output. The initial cardiac output in Subject 1 measured 3.84 liters per minute or 2.17 liters per minute per square meter of body surface (Table 1, Figure 1). Two hours after taking digitalis 0.9 gram the output remained unchanged; 4 hours afterward it fell to 3.68 liters per minute. Progressive decrease followed. The lowest point was recorded 24 hours later when it measured 2.54 liters per minute or 1.44 liters per minute per square meter of body surface, equal to 66 per cent only of its initial value. Twenty-four hours later still, the output was 3.01 liters per minute, that is to say, 81 per cent of the initial amount. From this time it returned slowly toward normal; one week later it was 3.40 liters per minute (88 per cent of normal) and after another it was still low.

The results in the 5 other persons were similar (Tables 1 and 2, Figures 2, 3, and 4), with this exception, that the effect of the drug was of shorter duration. The earliest effects were found after 4 to 12 hours; the maximum ones in 4 to 24 hours. At the time of the maximum effect the output was reduced to 60 to 85 per cent of the initial amount.

The normal resting arteriovenous oxygen difference in Subject 1 was 58.4 cc. per 100 cc. blood. Two hours after the administration of digitalis 0.9 gram it remained unchanged; four hours afterward a slight increase occurred (Table 1). Progressive increase took place so that 24 hours later it was 88.0 cc. per 100 cc. blood. The arteriovenous oxygen difference then returned slowly toward its initial value, although at the end of two weeks this had not been attained.

The results in the 5 other cases are similar to these except that the effect of the drug was of shorter duration and passed off within a shorter time (Tables 1 and 2). The maximum decrease in the arteriovenous oxygen difference was observed 4 to 24 hours after giving digitalis.

Effect on cardiac size. In Subject 1 the size of the heart measured 105.8 sq. cm. (Table 1, Figures 5 and 6). It decreased gradually after the administration of digitan 0.9 gram; it was smallest (97.2 sq. cm.) at the end of 15 hours measuring 91 per cent of its initial size. It was still small at the end of 24 hours, when the output was only 66 per cent of what it was at the beginning. It returned gradually toward its initial size although, as in the case of the cardiac output, this was not reached more than two weeks later.

The results in the 5 other cases corresponded to this one (Tables 1 and 2, Figures 2, 3, and 4). The decrease in size varied between 7 and 15 per cent (to 93 and 85 per cent of the initial measurement) and was recorded 4 to 24 hours after the drug was given. As the effect of the drug wore off the heart regained its original size.

Effect on cardiac rate. In all six subjects the cardiac rate fell 4 to 14 beats per minute, counted at the time the cardiac output was measured (Tables 1 and 2, Figures 1, 3, and 4). In each instance it returned toward the initial count as the effect of digitalis disappeared.

Effect on the electrocardiograms. In all instances change in form of the T-waves of the electrocardiograms occurred (Tables 1 and 2). In one instance (Subject 4) T_2 and T_3 which had been positive became diphasic and T_1, positive at the beginning, became negative (Table 1). In another instance (Subject 2) T_3 which was positive became negative; as the effect decreased it became diphasic and was diphasic still 38 days later when the last record was made (Table 1, Figure 7). It was upright one year later; when the observations were repeated T_3 was similarly affected (Table 2, Figure 7). In the four other subjects (Subjects 1, 3, 5, and 6) positive T_{123} became either smaller or isoelectric (Tables 1 and 2). Alterations of the T-waves occurred as early as two and a half hours after administration of the drug (Figure 7).

Increase in auriculoventricular conduction time did not occur nor did abnormal rhythms or premature contractions develop. In one instance (Subject 4) P_3 became negative, to resume its earlier form as the effect of digitalis declined and in another instance (Subject 1) the form of the P-waves was altered.

Effect on oxygen consumption. The oxygen consumption of each individual remained constant (Tables 1 and 2). The changes in cardiac output which were observed are, therefore, not attributable to alterations of oxygen consumption, but to changes in arteriovenous oxygen difference. The normal oxygen consumption of one subject (Subject 4) was diminished (Table 1) and remained approximately unchanged in estimations which were made later. There appeared

TABLE 1

Effect of Giving Digitalis on Cardiac Output and Cardiac Size in Normal Individuals

Subject number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output per cent of initial	Cardiac output	Cardiac area	Cardiac area per cent of initial	Cardiac rate	Cardiac output	Arterial pressure	Summary of effect on electrocardiogram	Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*per cent*	*liters per sq. m. per minute*	*sq.cm.*	*per cent*	*minutes*	*cc. per beat*	*mm. Hg*		*grams*	
1 ♂	34	March 4	66.9	168.5	1.77	224	58.4	3.84	100	2.17	105.8	100	80	48	112/68		0.9	Before
						226	58.5	3.87	100	2.20	103.4	98	73	53	120/72	T-waves became of lower voltage but remained positive. P-waves changed form. The ventricular rate became slower		2 hours after
						224	60.9	3.68	92	2.08	102.3	96	72	51	122/74			4 hours after
						224	74.0	3.03	81	1.71	102.8	97	72	42	132/64			9 hours after
						219	78.4	2.79	72	1.58	102.6	96	66	42	126/66			12 hours after
						217	75.1	2.89	75	1.63	97.2	91	72	40	120/70			15 hours after
		March 5	66.4		1.76	224	88.0	2.54	66	1.44	97.7	92	78	33	130/78			24 hours after
		March 6	66.2		1.76	221	73.4	3.01	81	1.71	100.4	94	78	39	130/80			48 hours after
		March 7	66.7		1.77	219	67.6	3.24	87	1.83	100.4	94	77	42	128/80			3 days after
		March 8	67.2		1.77	218	67.6	3.23	87	1.82	*		78	41	134/80			4 days after
		March 9	67.2		1.77	226	65.5	3.45	89	1.95	104.7	98	74	44	122/84			5 days after
		March 11	66.3		1.76	245	72.2	3.40	88	1.93	102.2	96	80	42	134/80			7 days after
		March 20	66.1		1.76	226	74.3	3.11	82	1.77	102.8	97	82	38	126/76			16 days after
2 ♂	31	February 16	73.9	175.5	1.89	239	62.2	3.84	100	2.03	110.5	100	58	66	95/60		0.8	Before
						242	64.7	3.74	98	1.98	107.4	97	58	64	108/70	T_3 became negative and was still diphasic on March 26, 1931. The ventricular rate became slower		4 hours after
						258	66.2	3.89	101	2.06	105.2	95	58	67	100/60			9 hours after
						251	74.1	3.38	88	1.78	101.5	91	59	57	100/65			12 hours after
		February 17	73.9			234	71.2	3.28	85	1.73	103.4	93	54	60	95/55			24 hours after
		February 18	74.1			233	59.1	3.94	103	2.08	112.5	101	58	68	90/50			48 hours after
		February 19	74.1			231	58.5	3.95	103	2.08	114.4	103	64	71	90/55			3 days after

TABLE 1—*Concluded*

Subject number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output per cent of initial	Cardiac output	Cardiac area	Cardiac area per cent of initial	Cardiac rate	Cardiac output	Arterial pressure	Summary of effect on electrocardiogram	Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*per cent*	*liters per sq.m. per minute*	*sq.cm.*	*per cent*	*minutes*	*cc. per beat*	*mm. Hg*		*grams*	
3 ♂	29	February 24	82.0	183.0	2.04	230	58.9	3.93	100	1.92	119.8	100	54	73	100/65		0.8	Before
						256	64.1	3.99	101	1.95	104.2	87	54	74	95/70	T_3 became of lower voltage but remained positive. The ventricular rate became slower		2 hours after
						224	53.7	4.17	106	2.04	110.5	92	50	83	90/70			4 hours after
						246	82.6	2.97	75	1.45	106.5	88	50	59	95/70			9 hours after
						230	56.6	4.06	103	1.99	104.6	87	50	81	90/60			15 hours after
		February 25	81.4		2.03	254	62.6	4.05	103	2.00	115.6	96	64	67	100/70			23 hours after
		February 26	82.0		2.04	229	53.8	4.23	108	2.07	112.7	94	60	71	100/75			48 hours after
		February 27	82.2		2.04	240	51.6	4.65	118	2.28	109.3	91	52	93	110/80			3 days after
4 ♂	31	June 16	61.6	165.5	1.69	169	63.8	2.65	100	1.57	136.3	100	70	38	120/90		1.0	Before
			61.6		1.69	143	68.9	2.08	79	1.23	124.0	90	66	32	135/90	T_{123} which were positive at beginning became—T_1 and diphasic T_{23}. The ventricular rate became slower. P_3 became negative and then diphasic		12 hours after
		June 17	60.9		1.68	163	87.6	1.86	70	1.11	120.0	88	60	31	120/90			24 hours after
		June 18	60.9		1.68	166	60.5	2.74	103	1.63	128.7	95	70	39	110/70			48 hours after

* Patient was not in true posterior-anterior position.

TABLE 2

Effect of Giving Digitalis on Cardiac Output, Cardiac Size, Venous Pressure, and Vital Capacity in Normal Individuals

Subject number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output per cent of initial	Cardiac output	Cardiac area	Cardiac area per cent of initial	Heart rate	Cardiac output	Arterial pressure	Venous pressure	Vital capacity	Body temperature (rectal)	Digitan	Summary of effect on electrocardiogram	Time with reference to digitalis administration
	years	*1932*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*per cent*	*liters per sq.m. per minute*	*sq.cm.*	*per cent*	*minutes*	*cc. per beat*	*mm. Hg*	*cm. blood*	*cc.*	*°F.*	*grams*		
5	29	January 26	68.7	170.7	1.79	238	60.3	3.65	100	2.04	95.6	100	68	54	108/70	5.0	4300	98.8			Before
♂																			1.0		
					1.79	231	73.5	3.14	86	1.75	93.8	98	58	54	100/50	6.0	4350	98.8		T_{123} became of lower voltage	4 hours after
					1.79	223	77.2	2.89	79	1.61	82.8	86	59	49	105/60	2.8	4200	99.2			9 hours after
					1.79	226	73.0	3.14	86	1.75	81.4	85	56	56	100/50	8.0	4300	99.0			14 hours after
		January 27	68.5		1.79	231	69.9	3.30	90	1.84	88.3	92	59	56	120/80	8.3	4350	98.8			24 hours after
		January 28	68.6		1.79	230	65.0	3.54	97	1.98	99.4	105	59	60	105/60	3.5	4350	98.6			48 hours after
																blood					
6	33	April 8	52.5	157.0	1.50	195	56.1	3.47	100	2.31	102.9	100	72	48	80/45	12.0	2700	98.8			Before
♀																			0.8		
						194	94.8	2.05	60	1.37	96.2	93	66	31	85/50	12.3	2700	99.2		T_{12} became of lower voltage and T_3 isoelectric	4 hours after
						187	78.2	2.39	69	1.59	98.6	96	70	34	85/50	11.2	2700	98.6			12 hours after
		April 9	52.7		1.51	198	85.9	2.31	67	1.53	96.7	93	72	32	85/45	11.2	2700	98.6			24 hours after
		April 10	52.7		1.51	186	68.0	2.73	79	1.81	97.7	94	72	38	100/60	12.6	2660				48 hours after
		April 11	53.2		1.51	201	76.0	2.65	77	1.76	100.7	97	75	35	98/60	13.0	2690				72 hours after
		April 13	53.4		1.51	202	68.0	2.97	86	1.97	104.2	101	76	39	100/55	13.0	2650				120 hours after

TABLE 2—*Concluded*

Subject number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output per cent of initial	Cardiac output	Cardiac area	Cardiac area per cent of initial	Heart rate	Cardiac output	Arterial pressure	Venous pressure			Vital capacity	Body temperature (rectal)	Digitan	Summary of effect on electrocardiogram	Time with reference to digitalis administration
	years	*1932*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*per cent*	*liters per sq.m. per minute*	*sq.cm.*	*per cent*	*minutes*	*cc. per beat*	*mm. Hg*	*cm. blood*			*cc.*	*°F.*	*grams*		
																"Blood up"	"Blood down"	"Salt down"					
2 ♂	32	April 18	72.2	179.5	1.89	258	60.6	4.26	100	2.25	111.5	100	58	73	80/45	19.0	22.3	23.5	4500	97.7			Before
																					1.0		
						254	69.4	3.66	86	1.95	106.4	95	55	67	80/45	18.0	23.0	21.7	4700			T_3 became diphasic and then negative	4 hours after
						259	71.7	3.61	85	1.91	95.8	86	56	64	90/50	18.0	21.0	19.0	4700				12 hours after
		April 19	71.7		1.89	249	73.2	3.40	80	1.80	100.9	90	50	68	80/40	22.0	23.0	22.2	4700				24 hours after
		April 22	71.6			260	62.6	4.15	98	2.20	111.5	100	58	72	80/40	22.0	23.0	23.0	4600				4 days after

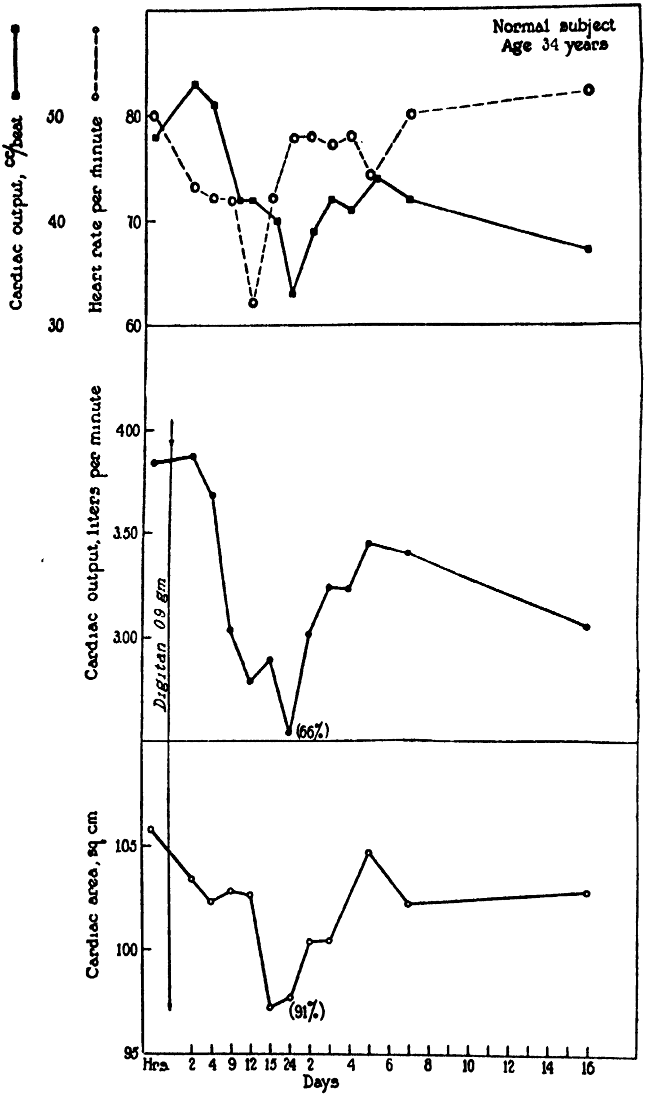


FIG. 1. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, and cardiac rate per minute in subject number 1.

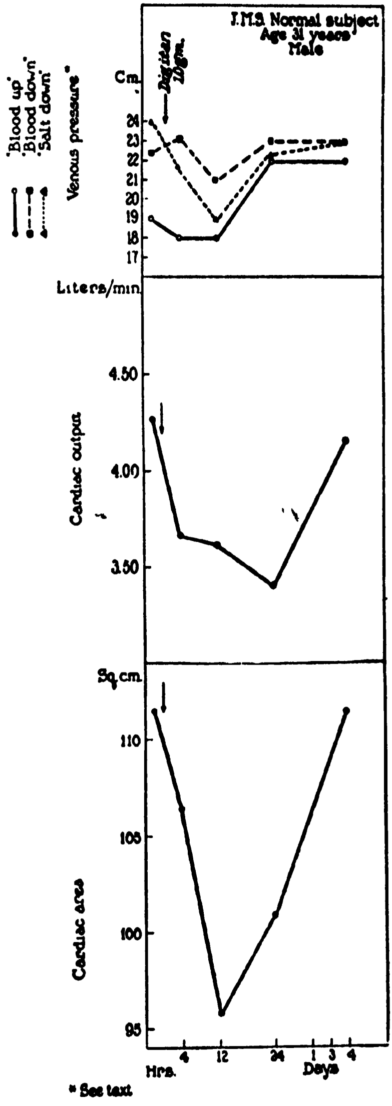


Fig. 2. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, and venous pressure in subject number 2.

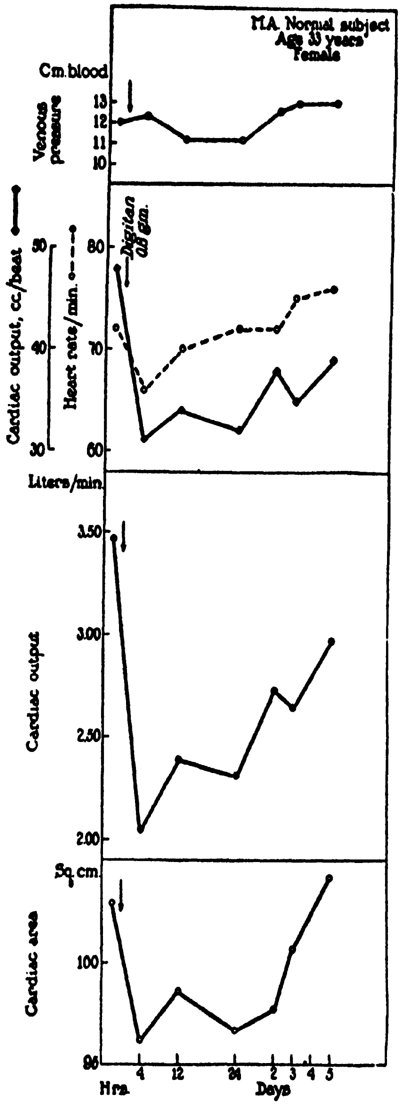


FIG. 3. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, cardiac rate, and venous pressure in subject number 6.

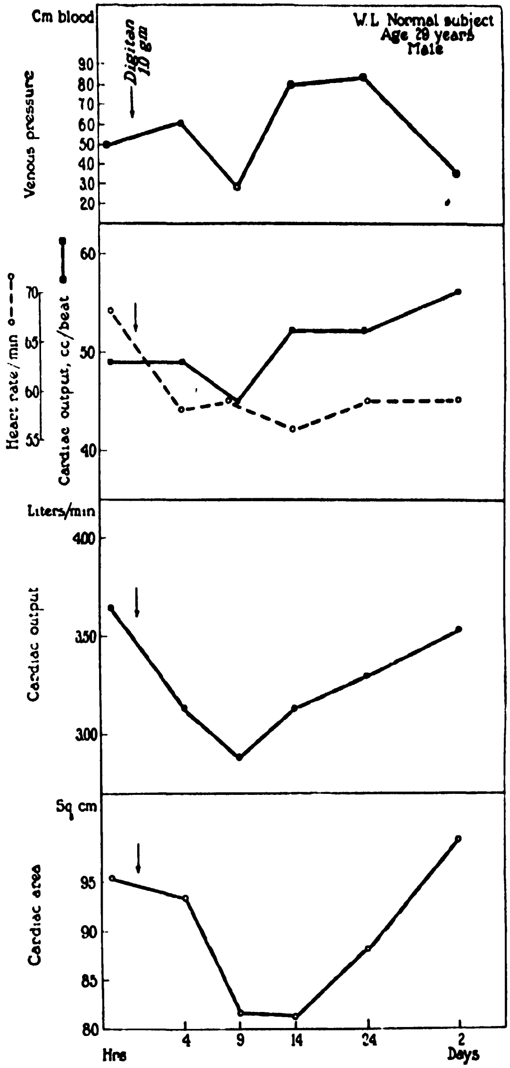


FIG. 4. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, and venous pressure in subject number 5.

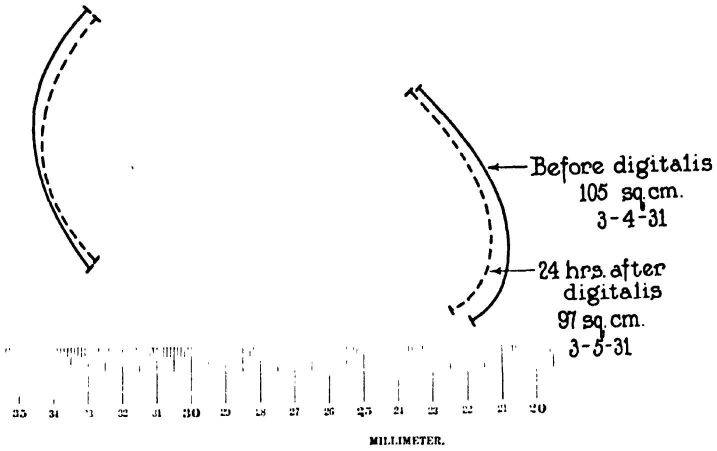
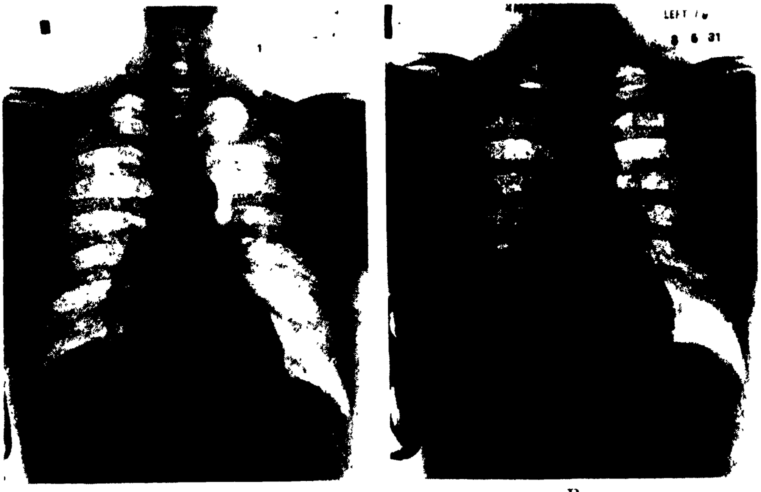


FIG. 5. In this figure is represented the change in size of the heart following the administration of digitan 0.9 gram in subject number 1.

The outlines of the heart were traced from the x-ray photographs taken on March 4 and 5 on thin paper and superimposed in the manner shown.



A B

FIG. 6. In this figure is represented the change in size of the heart following the administration of digitan 0.9 gram in subject number 1.

Photograph *A* was taken on March 4, 1931, immediately before and *B* on March 5, twenty-four hours after the administration of the drug.

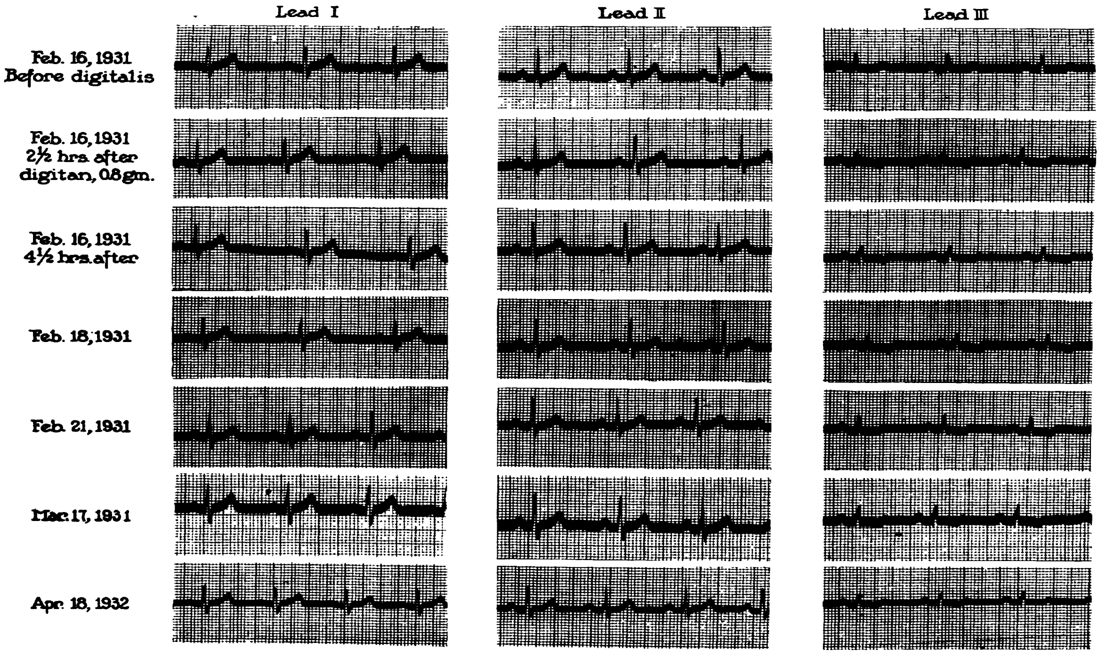

Lead I
Lead II
Lead III
Feb. 16, 1931 Before digitalis
Feb. 16, 1931 2½ hrs. after digitan, 0.8 gm.
Feb. 16, 1931 4½ hrs. after
Feb. 18, 1931
Feb. 21, 1931
Mar. 17, 1931
Apr. 18, 1932

FIG. 7. The effect of giving digitan 0.8 gram on the electrocardiogram of subject number 2 is shown in this figure.

Alterations of the T-waves in Lead III were present $2\frac{1}{2}$ hours after digitalis was given and were still present at the end of 38 days (March 17), when the observations were discontinued. It had, however, regained its usual contour one year later (April 18, 1932). Divisions of the ordinates equal 10^{-4} volts. Divisions of the abscissae equal 0.04 of a second. The original curves are sharply contrasted black and white; no half tones are lost by this method of reproduction. The electrocardiograms are reduced to one-half their natural size.

to be no adequate explanation for his low basal metabolic rate. Since the arteriovenous oxygen difference was normal, the initial cardiac output was less than it should have been for his height and weight (Grollman (12)). But after taking digitalis, as in the other subjects, decrease in cardiac output and in cardiac size took place.

Effect on vital capacity. Significant alterations did not occur in the vital capacities of the three individuals in whom it was measured (Table 2).

Effect on arterial and venous pressures. Significant alterations either in the systolic or diastolic level of the arterial blood pressure did not occur (Tables 1 and 2).

Estimations of venous pressure whether by direct or indirect methods are unsatisfactory especially from the point of view of comparing the results over long periods of time. Errors arise due to the method but also due to difficulty in selecting a level to represent that of the opening of the venae cavae into the right auricle, and in finding this again in making subsequent estimations. For these reasons the venous pressure was measured in two subjects (Subjects 5 and 6) by the method of Taylor, Thomas and Schleiter (18), but in a third (Subject 2) three methods were compared. In the former method the dry wall of the glass tube apparently hinders the rise of blood. An attempt to learn the pressure in the right auricle by taking the pressure elsewhere in the body is, of course, beset with difficulty. The changes which occur in any given region, independent of those elsewhere, make it impossible to be certain that a usual relation continuously exists between the pressures in two veins. It is reasonable to suppose, however, that the direction of change will not be uniformly different. In the absence of a direct method which gives the pressure in the right auricle, the one that has been used yields perhaps the best approximation.

The third subject lay flat on a wide board without pillows at least 15 minutes, his arms at his sides. His venous pressure was measured with the following apparatus. The two arms of a Y-tube supplied near their origins with stopcocks were bent at right angles to form manometers; the third one was connected to a needle (Figure 8). The apparatus was sterilized. One of the tubes having a funnel at

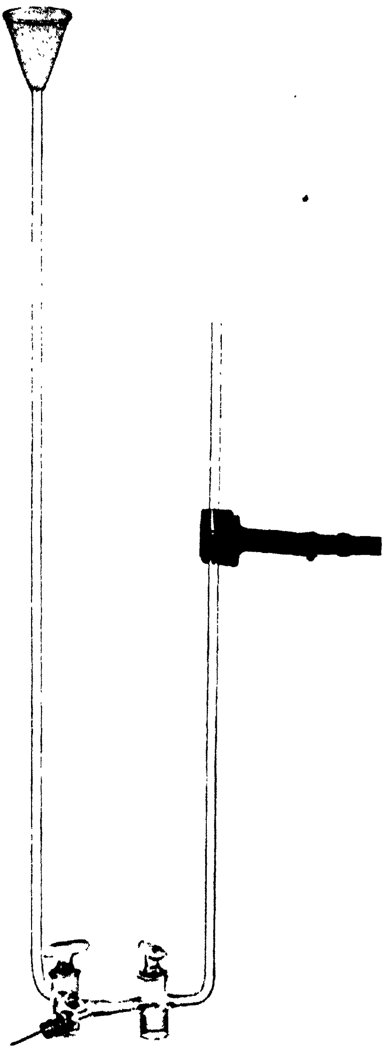


FIG. 8. In this figure is represented a photograph of the apparatus which was used in the measurement of venous pressure in subject number 2 (table 2).

its upper end was filled with warm sterile normal saline solution, its stopcock being closed. The needle was inserted into a vein. Blood flowed into the open tube. The height of the column was measured from the level of the board ("blood up"). By manipulation or by using a tourniquet, more blood was forced into the manometer. On flowing back into the vein the column came to rest ("blood down"). This stopcock was then closed and the one connected to the tube containing salt solution opened. When this column came to rest, its height also was measured ("salt down"). The needle was always inserted at the same point in the same vein.

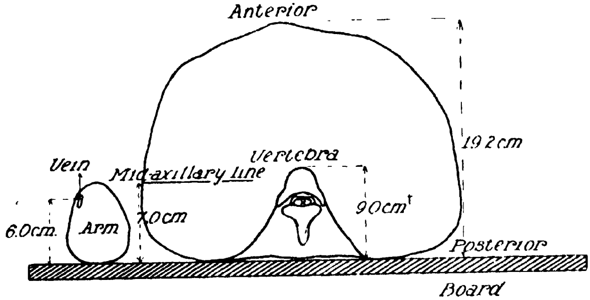


FIG. 9. In this figure is shown the position of the arm during measurements of venous pressure in subject number 2 (Table 2).

The anatomical distances are given.

† This measurement was made from an x-ray photograph of the chest (lateral view) taken at a distance of 2 meters.

Venous pressure estimated in these three ways showed wide variations (Table 2, Figure 2). The height taken by the "blood up" method was usually lower than by the "blood down," and "salt down" greater than the "blood up," but sometimes it was greater and sometimes less even than the "blood down" measurement. All measurements were made from the level of the board on which the subject lay. To find the correct pressure, a correction must be made to allow for the height of the caval openings above the board. To learn this, x-ray photographs are taken at 2 meters, the subject lying flat on his back (Figure 9). It appears that the height sought is approximately 11.0 to 12.0 cm. This, or a similar appropriate figure (found in each case), must be subtracted from the manometric value. If the initial "blood up" measurement is selected, the venous pressure is 19.0 cm.; if the "blood down," 22.3 cm.; if the "salt down," 23.5 cm. (Subject 2).

Detailed attention was given to the matter of the level of venous pressure because it has attained importance from the position taken by Tainter and Dock (4) who believe that decrease in cardiac output in normal dogs after giving digitalis is due to constriction of the hepatic veins. The venous pressure, they found, was low. In the three human cases studied when alterations in cardiac minute output are correlated with the height of the venous pressure, it appears from estimations made that no consistent change was observed.

In one instance (Subject 5) the minute output diminished to 86 per cent of its normal amount four hours after giving digitalis (Table 2, Figure 4), the venous pressure having increased 1.0 cm. (from 5.0 to 6.0 cm.). At the end of nine hours when the output fell to 79 per cent, the venous pressure measured 2.8 cm., although fourteen hours afterward when the output was again 86 per cent the pressure was high (8.3 cm.). Forty-eight hours afterward, when the minute output was normal (97 per cent), the venous pressure was low (3.5 cm.). In a second patient (Subject 6, Table 2, Figure 3), the variations of venous pressure were smaller, ranging from 0.8 cm. below to 1.0 cm. above the initial measurement, though the output fell to 60 per cent. In a third patient (Subject 2, Table 2, Figure 2), the three methods of taking venous pressure gave inconsistent readings at the four hour period. When the output was near its lowest point, the pressure by all methods was low, but when it was actually lowest, they were again high, two of them above their initial values.

The effect on symptoms. The symptoms of one individual (Subject 1) deserve detailed description. Twelve to fifteen hours after taking digitalis he became conscious that his heart was beating slowly and forcefully, so that he felt distress in his cardiac region. There was moderate dyspnea while lying in bed on two pillows. These symptoms were relieved slightly when he sat in a half-reclining position. The cardiac minute output at this time had fallen to 70 per cent. Twenty-four hours afterward, when he began again to walk about, more than the slightest exertion was impossible without creating severe dyspnea. He was, in fact, comfortable only when sitting upright at rest. At this time the output was 66 per cent and the size of the heart smaller than at the beginning. At thirty-six hours, dyspnea became much more severe, so that walking as slowly as possible elicited severe dyspnea and distinct cardiac pain. The pain did not radiate. The sensation was as if the heart were being squeezed. He was forced to rest two or three times in walking the length of a city block to recover from shortness of breath and to allow the cardiac pain to pass off. The next day these symptoms were still present but to a less extent. They persisted for several weeks. The duration of the symptoms corresponded to the period of diminished cardiac output. Associated with diminished cardiac output, decrease of the volume flow of blood through the coronary arteries must, in all probability, have occurred, and in consequence a degree of anoxemia on the part of the muscle. Were this the train of events, the inference is not unjustified that pain, which was experienced in so prominent a form, was the result of cardiac ischemia, in accordance with the view which has recently been revived by Keefer and Resnik (19).

Three other subjects complained of lassitude in the second twenty-four hours after digitalis was given. They were able to engage, in spite of low outputs, in their usual activities. One (Subject 2) complained of dyspnea on climbing a flight of stairs when this output was low, but recovered when it returned to normal. The other two complained of no disagreeable symptoms.

SUMMARY AND CONCLUSIONS

The effect of giving digitalis to six normal individuals was studied with particular reference to its effect on cardiac output, on cardiac size, and on venous pressure. The following phenomena were observed: (1) The output of blood from the heart decreased. (2) The size of the heart diminished. (3) Slight decrease in cardiac rate occurred. (4) These effects were at a maximum four to twenty-four hours after giving the drug. (5) Significant changes did not occur in the levels of arterial and venous pressure. (6) Changes in form, sometimes slight, of the T-waves of the electrocardiogram occurred in each instance and were present as early as 2½ hours after the drug was given. (7) As the effect of digitalis wore off (48 hours to 3 weeks), output, size, rate, and the T-waves of the electrocardiogram returned toward their initial values. (8) A correlation was not established from these data between decrease in cardiac output and change in the level of venous pressure.

PART II

The Effect on the Output of Hearts in Heart Failure with Congestion, in Human Beings

It has been demonstrated as a fact that giving digitalis decreases the cardiac output and cardiac size in normal human beings. What the effect of its administration is when hearts are in a state of failure is still unknown. Investigations to discover this have, therefore, been undertaken and are now reported.

Seven patients exhibiting signs and symptoms of heart failure were studied. Although Grollman, Proger, and Dennig (11) have shown that the acetylene method is adequate for the analysis of cardiac patients exhibiting pulmonary stasis, since the gases in a rebreathing bag come into equilibrium with the blood leaving the lungs within a prescribed time, only such cases were selected as were to all intents and purposes free of pulmonary congestion. The lungs were usually clear at the time the measurements were made. Rarely, at the extreme bases behind, a very few râles were heard on deep breathing.

Methods

All the patients when studied were at rest in bed. They were taking ward diet, free of salt except that used in cooking. The intake of fluid was, except in the case of one patient, limited to 1200 cc. a day. In the excepted case it was not limited. Measurements of cardiac output were made as in normal persons by the acetylene method (9, 10, 11). Owing to the difficulty cardiac patients experience in breathing when recumbent, all observations were made one half-hour after assuming the sitting position (20) at an angle of 135 degrees, the legs being fully extended. They were made familiar with the procedures beforehand. The vital capacity of the patients was estimated and the volume of gas in the bag adjusted to that amount which each, within a given time, could mix completely (9, 10, 11). This adjustment is necessary because it is impossible, during dyspnea when the vital capacity is diminished, for patients to manage an amount of gas (2400 cc.) as great as can normal individuals.

The size of the heart, in x-ray photographs, was measured as before (13). Electrocardiograms were made; the heart rate was counted; the arterial blood pressure and the vital capacity were estimated. In certain instances venous blood pressure was measured by the method of Taylor, Thomas, and Schleiter (18). The observations were made in the following order: the patients, in a *basal metabolic state*, rested in the sitting position while the radial pulse (and cardiac rate in the case of patients exhibiting fibrillation of the auricles) was counted at intervals of five minutes. At the end of one half-hour they rebreathed the acetylene-air mixture. Samples of gas were taken for estimation of the arteriovenous oxygen difference, the first after rebreathing 7 to 8 times within fifteen seconds, the second after 3 to 4 additional rebreathings within ten seconds, the samples being taken during expiration. Shortly afterward the oxygen consumption was measured with a Benedict-Roth spirometer. After a pause of a few minutes the arterial and venous pressures and the vital capacity were measured in succession, and electrocardiograms were taken. Then x-ray photographs were secured.

Observations were made immediately before the administration of digitalis and at frequent intervals afterward. Digitalis in the form of digitan (Merck) 1.0 gram was given either in a single dose or within ten hours; in one instance, the dose was 0.8 gram, and in another 1.3 grams. Nausea and vomiting did not occur.

OBSERVATIONS

Four patients exhibiting normal cardiac mechanism in the presence of signs and symptoms of heart failure comprise the first group.

Case 1. D. T. T. (Hospital number 7696), a Negro, 47 years of age, was admitted to the hospital on March 12, 1931. He was the subject of high systolic pressure (195 mm. Hg), discovered at the age of 43 years during a physical examination to which he submitted because of the occurrence of temporal headaches. At the age of 46 years he suffered from an attack of rheumatic fever and pericarditis. Two months later dyspnea appeared. Three months later still, he began to suffer from attacks of nocturnal dyspnea occurring at intervals of two weeks. Two months before admission dyspnea became constant and six weeks later, edema of the legs first appeared. On admission he complained of palpitation. He was dyspneic. A few râles were heard at the bases of the lungs posteriorly but disappeared within three days. There was general arteriosclerosis; the systolic blood pressure measured 170 mm. Hg, the diastolic 100 mm. Hg; pulsus alternans was present; the heart was enlarged; its rhythm was normal; there were signs of mitral insufficiency. The liver was enlarged and there was ascites; edema of the extremities, although present on admission, disappeared after resting in bed three days. The *etiological* diagnosis[5] was: rheumatic fever, arteriosclerosis, arterial hypertension; the *anatomical*: cardiac hypertrophy, mitral insufficiency, left ventricular preponderance; the *physiological*: normal sinus rhythm, heart failure of the congestive type (first attack), pulsus alternans.

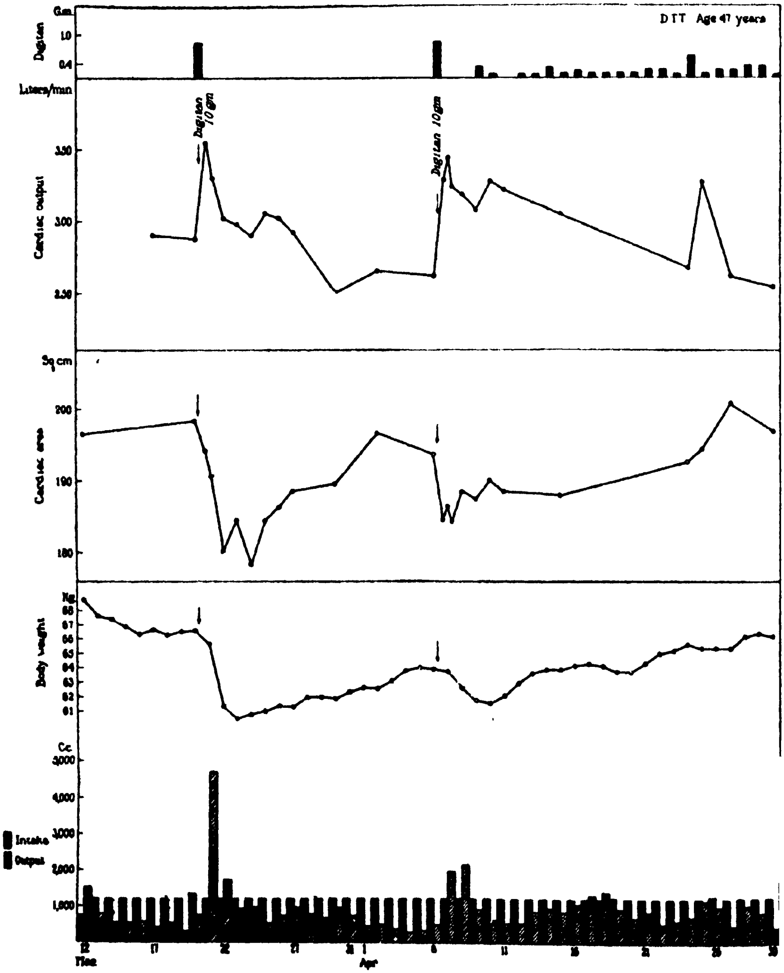
His cardiac output during the period of heart failure measured 2.90 liters per minute[6] on March 17, and 2.88 liters, three days later (March 20) (Table 3, Figure 10A). The area of the heart equalled 198.3 sq. cm. (Table 3, Figures 11 and 12). When digitalis (digitan 1.0 gram) was given (March 20) diuresis occurred (Figure 10A), he lost weight, became free of dyspnea and palpitation, and the liver became smaller (March 25). On March 21, 15 hours after taking the drug, the cardiac output increased to 3.54 liters per minute and the size of the heart decreased to 180.2 sq. cm. (March 22); slowing of the cardiac rate and alterations of the form of the T-waves of the electrocardiograms occurred (Table 3, Figures 10A, 11, and 12). As the effect of digitalis wore off the signs of heart failure reappeared (dyspnea, palpitation, enlargement of the liver). Two weeks later (April 6) the cardiac output fell to 2.62 liters per minute and the size of the heart increased to 193.6 sq. cm. When digitalis was given on three other occasions, April 6, May 11, and June 12, results similar to those just described were observed; increase in diuresis and in cardiac output, and decrease in cardiac size. As the effect of digitalis declined, the signs of heart failure reappeared; output decreased and cardiac size increased (Table 3, Figures 10A and 10B).

Summary. In this patient exhibiting normal cardiac mechanism in the presence of signs and symptoms of heart failure of the congestive type, the volume output of blood from the heart was diminished and the heart was large. On administering digitalis on each of four occasions, output increased and the size of the heart diminished. Changes in the reverse fashion occurred as the effect of digitalis disappeared. Giving digitalis induced diuresis on each occasion and was followed by amelioration of his signs and symptoms.

---

[5] The diagnoses in this paper conform to the nomenclature for cardiac diagnosis recommended by the Heart Committee of the New York Tuberculosis and Health Association. "Criteria for the classification and diagnosis of heart disease." 2d ed. New York Tuberculosis and Health Association, New York. 1929.

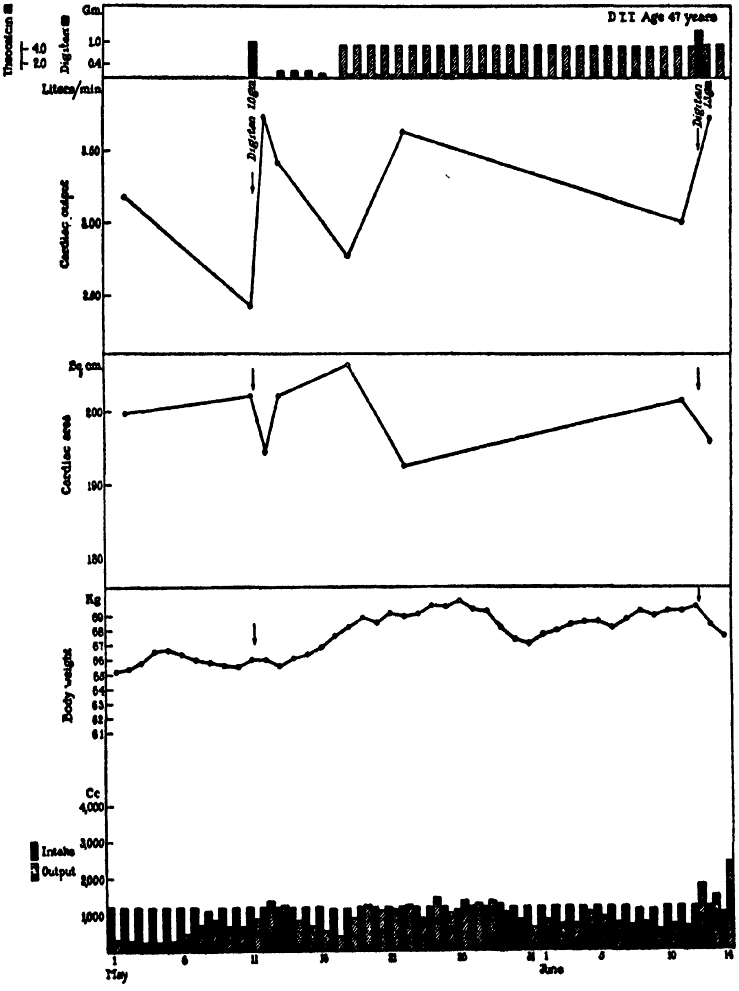
[6] In the description of events and in the figures, the total cardiac output per minute is recorded. Calculation of the cardiac output on the basis of the surface area of the body (liters per minute per square meter of body surface) frequently showed the changes in a more striking manner, especially if diuresis occurred and the patient lost weight. These measurements are included in the tables (Tables 3 and 4).



A

See legend on opposite page

Case 2. M. R. (Hospital number 7852), a Negro aged 33 years was admitted to the hospital on September 22, 1931. He enjoyed good health until he "caught cold" four months before admission and suffered from shortness of breath for a



B

Fig. 10 *A* and *B*. In these figures is represented the effect of giving digitalis on cardiac output, cardiac size, and volume of urine in D. T. T. (Case 1).

The cardiac mechanism was normal during heart failure.

TABLE 3

Effect of Giving Digitalis on Cardiac Output and Cardiac Size in Patients Exhibiting Normal Cardiac Rhythm in the Presence of Signs and Symptoms of Heart Failure

Case and hospital number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output	Cardiac area	Cardiac rate	Cardiac output	Arterial pressure	Vital capacity	Theocalcin	Effect on T-wave of electrocardiogram			Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*liters per sq.m. per minute*	*sq.cm.*	*minutes*	*cc. per beat*	*mm. Hg*	*cc.*	*grams*	T_1	T_2	T_3	*grams*	
1	47	March 17	66.5	165.3	1.74	306	105.3	2.90	1.66	195.4	88		¶			−	−	0		Before
D. T. T.		March 20	66.5		1.74	295	102.2	2.88	1.65	198.3	108	27	180–150/125			−	−	∓		
7696		March 20																	1.0	
♂		March 21	65.6		1.73	292	82.5	3.54	2.00	194.1	88	40	175–160/120			∓	∓	∓		15 hours after
			65.6		1.73	327	99.0	3.30	1.91	190.7	108	31				+	∓	−		22 hours after
		March 22	61.4		1.67	292	97.1	3.01	1.80	180.2	104	29	180–145/120			+	∓	−		2 days after
		March 23	60.5		1.66	271	90.9	2.98	1.80	184.5	98	30	160–140/110			+	∓	−		3 days after
		March 24	60.8		1.67	273	94.0	2.90	1.74	178.4	88	33	165–135/110			∓	∓	−		4 days after
		March 25	61.0		1.67	258	84.5	3.05	1.83	184.4	103	30	140–130/110			∓	∓	−		5 days after
		March 26	61.3		1.68	247	81.6	3.03	1.80	186.4	92	33	145–140/110			∓	∓	0		6 days after
		March 27	61.3		1.67	264	90.5	2.92	1.75	188.6	92	32	150–135/110			∓	∓	+		7 days after
		March 30	61.9		1.68	259	103.6	2.50	1.50	189.5	100	25	170–130/110			−	∓	+		8 days after
		April 2	62.6		1.69	297	111.7	2.66	1.57	196.5	96	23	180–130/120			−	∓	+		
		April 6	64.0		1.71	304	115.8	2.62	1.53	193.6	96	25	175–140/130			−	∓	+		Before
		April 6																	1.0	
		April 7	63.8		1.71	286	87.0	3.29	1.92	184.5	100	33	175/130			∓	∓	−		9 hours after
			63.5		1.71	289	84.0	3.44	2.01	186.5	96	36	175–140/120			∓	∓	−		15 hours after
			63.6		1.71	231	89.8	3.24	1.89	184.3	96	34	160–140/120			+	∓	−		23 hours after
		April 8	62.6		1.69	303	95.1	3.19	1.89	188.5	84	38	150–130/105			+	∓	−		2 days after
		April 9	61.8		1.68	296	96.4	3.07	1.83	187.5	98	31	175–140/118			∓	∓	∓		3 days after
		April 10	61.6		1.68	290	88.4	3.28	1.95	190.0	100	33	175–150/120			∓	∓	∓	1.0	4 days after

TABLE 3—*Continued*

Case and hospital number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output	Cardiac area	Cardiac rate	Cardiac output	Arterial pressure	Vital capacity	Theocalcin	Effect on T-wave of electrocardiogram			Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*liters per sq.m. per minute*	*sq.cm.*	*minutes*	*cc. per beat*	*mm. Hg*	*cc.*	*grams*	T_1	T_2	T_3	*grams*	
		April 11	62.2		1.68	278	86.0	3.23	1.92	188.7	98	33	152–135/110			∓	∓	∓		5 days after
		April 15	63.9		1.71	256	84.2	3.05	1.78	188.0	94	32	170–140/115			∓	∓	∓		
		April 16																	1.1 (April 16–23)	
		April 24	65.6		1.74	287	107.1	2.67	1.53	192.4	104	25	178–130/120			∓	∓	∓	0.6	
		April 25	65.4		1.73	311	95.1	3.27	1.89	194.3	96	34	180–150/115			∓	∓	∓		
		April 27	65.4		1.72	265	101.7	2.61	1.51	200.7	96	27	170–140/120			∓	∓	+	1.2	
		April 30	66.2		1.73	264	103.7	2.54	1.47	196.5	96	26	190–150/120			∓	∓	0		
		May 1																	Discontinued	
		May 2	66.4		1.74	296	93.4	3.17	1.82	199.7	94	33	180–150/115			∓	∓	∓		
		May 11	67.0		1.74	263	108.1	2.43	1.39	202.2	97	25	160–130/110			+	–	–	0.7	
		May 12	67.0		1.74	265	70.9	3.74	2.15	194.6	92	40	150–145/110			+	∓	–		
		May 13	66.6		1.74	277	81.1	3.41	1.96	200.2	104	32	175–155/115	2200					0.7 (May 13–16)	
		May 18	69.2		1.76	308	111.0	2.77	1.57	206.6	110	25	170–140/120	2375	4.5 q.d.	∓	∓	+	0.1 q.d. (May 18–30)	
		May 22	70.0		1.77	290	79.6	3.64	2.05	194.7	100	36	160–150/110	2400		∓	∓	+		
		May 30														Discontinued			Discontinued	
		June 11	70.4		1.77	300	99.6	3.00	1.69	201.5	98	30	160/120	1940		–	–	0		Before
		June 12																1.3	1.3	
		June 13	69.4		1.76	307	82.4	3.72	2.11	195.9	91	40	140–120/110	2000		–	–	–		24 hours after

TABLE 3—*Continued*

Case and hospital number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output	Cardiac area	Cardiac rate	Cardiac output	Arterial pressure	Vital capacity	Theocalcin	Effect on T-wave of electrocardiogram	Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*liters per sq.m. per minute*	*sq.cm.*	*minutes*	*cc. per beat*	*mm. Hg*	*cc.*	*grams*	T_1 T_2 T_3	*grams*	
2 M. R. 7852 ♂	33	September 23	49.8	156.0	1.47	224	109.4	2.05	1.39	143.4	74	28	150/100	1200		+ ∓ ∓		Before
		September 23															1.0	
		September 24	49.3		1.46	203	74.3	2.73	1.87	130.4	64	43	170/90	1275		+ ∓ ∓†		24 hours after
		September 25	49.0		1.46	200	81.9	2.44	1.67	134.9	62	40	150/100	1325		∓ ∓ ∓‡		48 hours after
		September 29	46.4		1.42	185	66.7	2.77	1.95	132.4	70	40	150/100	1300		− − −		
		October 5	45.7		1.42	186	128.8	1.44	1.01	134.2	76	20	160/100	1300		± − − less negative		
		October 6															1.0	
		October 7	45.4		1.41	197	89.1	2.21	1.57	125.8	74	30	165/100	1300		+ ∓ ∓		24 hours after
		October 8	45.4		1.41	203	101.3	2.00	1.42	134.4	76	26	165/100	1275		+ ∓ ∓		48 hours after
		October 9	45.3		1.41	202	123.0	1.64	1.16	139.6	76	22	160/100	1250		± ∓ ∓		3 days after
		October 13	46.1		1.42	194	118.7	1.63	1.15	144.5	84	20	160/100	1125		Change in reverse direction		
		October 14													2.0			
		October 15	46.0		1.42	173	71.3	2.42	1.70	136.4	72	34	145/95	1450	4.5			
		October 16	44.6		1.41	202	65.8	3.07	2.17	128.8	70	44	145/95	1325	q.d.			
		October 19	43.9		1.38	197	64.8	3.04	2.20	135.2	66		140/90	1500				
		October 20	44.1		1.40	205	60.3	3.40	2.43	132.3	66	52	140/90	1700				

TABLE 3—*Concluded*

Case and hospital number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output	Cardiac area	Cardiac rate	Cardiac output	Arterial pressure	Vital capacity	Theocalcin	Effect on T-wave of electrocardiogram			Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*liters per sq.m. per minute*	*sq.cm.*	*minutes*	*cc. per beat*	*mm. Hg*	*cc.*	*grams*	T_1	T_2	T_3	*grams*	
3	32	March 27	62.5	178.0	1.78	303	122.9	2.46	1.38		116	21	120/80			+	+	+		
W. N.		March 28	62.1		1.78	290	111.1	2.61	1.46	248.9	108	24	120/80			+	+	+		
7712		March 30	62.5		1.79	283	104.1	2.72	1.52	252.7	122	22				+	+	+		Before
♂		March 30																	1.0	
		March 31	63.1		1.79	273	103.0	2.65	1.48	247.7	80*	33	125/60			+	+	+		13 hours after
			63.4		1.78	260	103.0	2.52	1.41	247.7	74**	34	125/60			−	+	+		23 hours after
		April 1	63.4		1.78	278	109.1	2.55	1.42	237.4	88§	28	120/70			±	+	+		2 days after
		April 2	63.7		1.79	276	147.7	1.87	1.04	236.7	88*	21	125/70			−	+	+		3 days after
4	21	June 1	39.5	162.0	1.38	179	81.8	2.19	1.59	141.4	84	26	100/55	2200		+	+	+		Before
C. M.		June 1																	1.0	
7779		June 2	39.6		1.38	191	63.5	3.00	2.17	133.7	80	40	80/50	2300		+	+	+ all lower voltage		24 hours after
♀		June 3	39.2		1.37	207	75.5	2.74	2.00	134.8	80	38	98/55	2300	•	+	+	+ all lower voltage	0.2	48 hours after
		June 4	39.1		1.37	189	70.6	2.68	1.96	129.9	84	32	105/50	2425		+	+	+ all lower voltage		72 hours after

¶ Pulsus alternans was present; the two systolic readings represent the levels at which the alternate beats came through.

† Incomplete heart block; occasional 2:1 block; conduction time increased.

‡ Incomplete heart block has disappeared.

* Coupled rhythm due to ventricular premature contractions, right; ventricular premature contractions were felt in the radial pulse.

** Not coupled.

§ Ventricular premature contractions every 2d, 3d or 4th beat; ventricular premature contractions were felt in the radial pulse.

few days. This experience was repeated two months later. On admission he complained of shortness of breath. The heart was enlarged; there were signs of mitral insufficiency and aortic roughening. There were a few râles at the bases of the lungs posteriorly. X-ray photographs of the chest showed that there was a small collection of fluid in the space between the upper and lower lobes of the right lung. The liver was enlarged. Ascites and edema of the extremities were present. The systolic blood pressure measured 150 mm. Hg; the diastolic, 100 mm. Hg. The Wassermann reaction in the blood was positive. The *etiological* diagnosis was syphilis; the *anatomical*: cardiac hypertrophy, mitral insufficiency, aortic roughening, left ventricular preponderance; the *physiological*: normal sinus rhythm, slight arterial hypertension, heart failure of the congestive type (first attack).

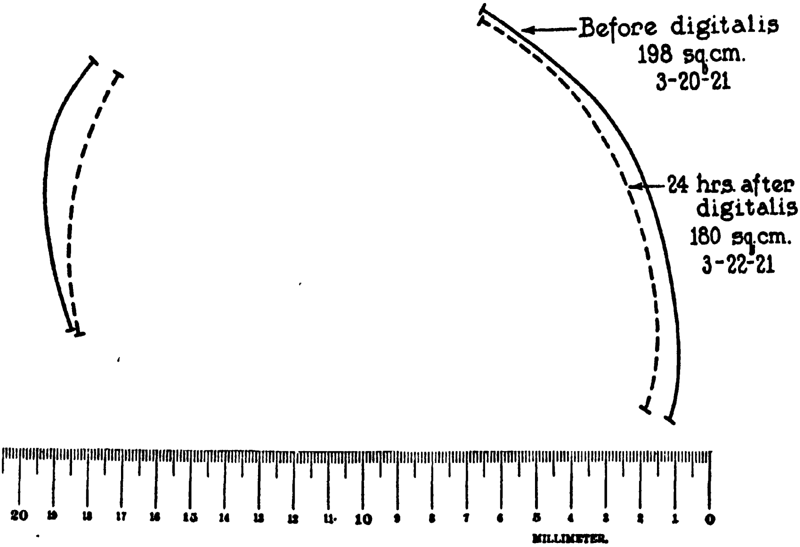
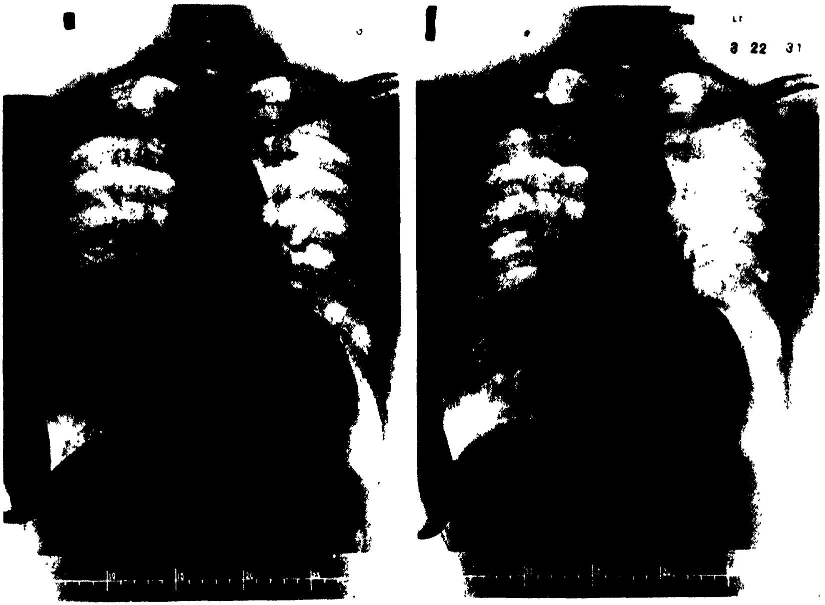


FIG. 11. In this figure is demonstrated the decrease in size of the heart which occurred following the administration of digitan 1.0 gram in D. T. T. (Case 1).

The outlines of the heart were traced on thin paper from the x-ray photographs.

Diuresis occurred promptly when digitalis (digitan 1.0 gram) was administered on September 23. He lost weight, edema, ascites, and râles disappeared, the liver became smaller, dyspnea became less, and the interlobar fluid diminished. The cardiac output which had been less than normal, 2.05 liters per minute, increased in 24 hours to 2.73 liters, and the size fell to 130.4 sq. cm. from having been 143.4 sq. cm. (Table 3, Figure 13). Vital capacity increased and cardiac rate became slower; alterations in the T-waves of the electrocardiogram occurred.

On September 29 the output measured 2.77 liters per minute and the heart was smaller than in the state of decompensation. As the effect of digitalis wore off he became dyspneic, the interlobar fluid re-collected, the liver increased in size. Ten days later (October 5) the cardiac output was only 1.44 liters per minute and the heart was larger, 134.2 sq. cm. Digitan 1.0 gram was given a second time (October 6), but diuresis failed to occur. He became, however, less dyspneic. Next day there was a temporary rise in the cardiac output (2.21 liters per minute) and decrease in cardiac size (125.8 sq. cm.). He became more dyspneic, the liver increased in size, ascites appeared, and on October 13 the output fell to 1.63 liters, and cardiac size rose to 144.5 sq. cm. Administration of theocalcin was then begun. Diuresis occurred promptly and he became free of edema. Increase in cardiac output occurred again (2.42 liters per minute, October 15) as well as decrease in cardiac size (128.8 sq. cm.), and increase in vital capacity. These relations remained unchanged for the next five days. Observations were then discontinued.



A B

FIG. 12. In this figure are reproduced x-ray photographs obtained in the case of D. T. T. (Case 1).

A was taken on March 20, before, and *B* on March 22, twenty-four hours after digitalis had been given.

Summary. The cardiac output in this patient exhibiting normal cardiac mechanism was diminished during heart failure and the heart was larger than in the state of compensation. On the administration of digitalis, cardiac output increased, cardiac size diminished, vital

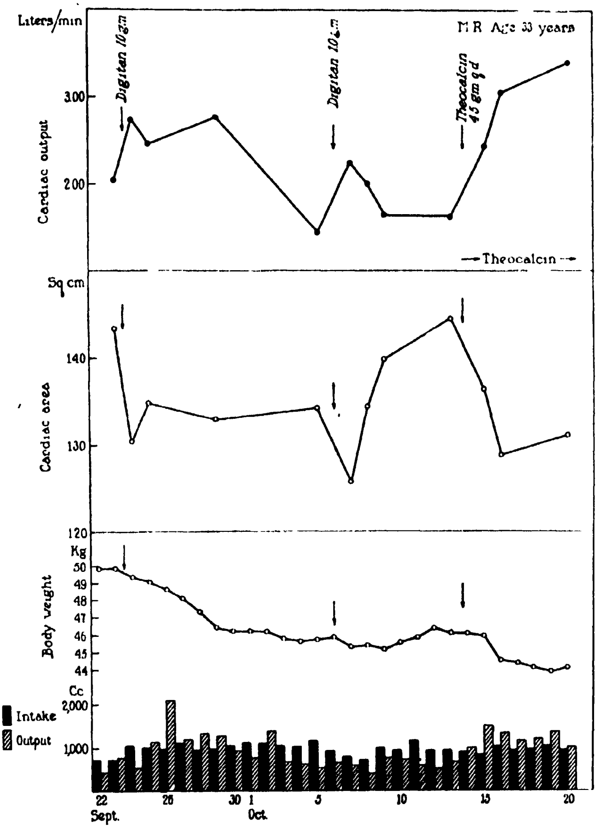


FIG. 13. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, and volume of urine in M. R. (Case 2).

The rhythm was normal during heart failure.

capacity increased slightly, alterations in the form of the T-waves of the electrocardiograms occurred, and the patient became free of the signs of heart failure. Changes occurred in the reverse fashion as the effect of the drug wore off. These events were repeated to a lesser degree on a second administration of digitalis though diuresis did not occur. But when theocalcin was given, diuresis took place, the output increased, and the size of the heart decreased to an amount greater than resulted from giving digitalis.

Case 3. W. N. (Hospital number 7712), a white man aged 32 years, was admitted to the hospital on March 26, 1931. He suffered from attacks of rheumatic fever at 17, 18, and 29 years of age. His heart was affected during the first attack. Shortness of breath beginning suddenly when he was 28 years of age incapacitated him for two weeks; afterward dyspnea was present on exertion. Four months before admission dyspnea increased and edema of the extremities appeared. One month later he stopped work. Digitalis was prescribed and he became free of edema. Recently, taking the drug induced ventricular premature contractions; coupled rhythm resulted. He discovered that breathing deeply or coughing usually relieved him of this rhythm. On admission he was cyanotic, there were a few râles at the bases of the lungs posteriorly, the pleural cavities were free of fluid, ascites and edema of the extremities were present, and the liver was swollen. The heart was enlarged, its rhythm regular. There were signs of mitral stenosis and insufficiency and of aortic insufficiency. The *etiological* diagnosis was: rheumatic fever; the *anatomical*: cardiac hypertrophy, aortic insufficiency, mitral stenosis and insufficiency, right ventricular preponderance; the *physiological*: regular sinus rhythm, ventricular premature contractions, heart failure of the congestive type (first attack).

On March 30, the heart's output was low (2.72 liters per minute) and its size large (252.7 sq. cm.) (Table 3). On administration of digitalis (digitan 1.0 gram) (March 30) coupled rhythm due to ventricular premature contractions occurred and persisted. Diuresis did not take place. He gained weight. Twenty-four hours after taking digitalis (March 31) the cardiac output remained approximately unchanged but the size of the heart decreased slightly. Seventy-two hours later, the cardiac output fell to 1.87 liters per minute. The rhythm was still coupled.

Summary. In this instance digitalis was without diuretic effect. Although the size of the heart became smaller, cardiac output was at first unchanged, though it diminished later. Coupled rhythm occurred after digitalis was given, and persisted. Alterations in the form of the T-waves of the electrocardiograms occurred.

The effect of giving digitalis when the rhythm of the heart is normal and when there are *no signs* of heart failure of the congestive type was shown in the case of C. M. (*Case 4*, Hospital number 7779), a white girl who was admitted to the hospital May 22, 1931, complaining of dyspnea. She suffered attacks of rheumatic fever at ages 5, 6, 7, 8, 9, and 12 years. When first examined at this hospital at 9 years of age, involvement of the heart had already occurred. Dyspnea first appeared when she was 13 years of age. On admission to the hospital at the age of

21 years the heart was enlarged; there were signs of mitral stenosis and aortic insufficiency. Dyspnea was present. There were *no signs* of heart failure of the congestive type. The *etiological* diagnosis was: rheumatic fever; the *anatomical*: cardiac hypertrophy, mitral stenosis, aórtic insufficiency, right ventricular preponderance; the *physiological*: normal sinus rhythm, ventricular premature contractions, cardiac insufficiency.

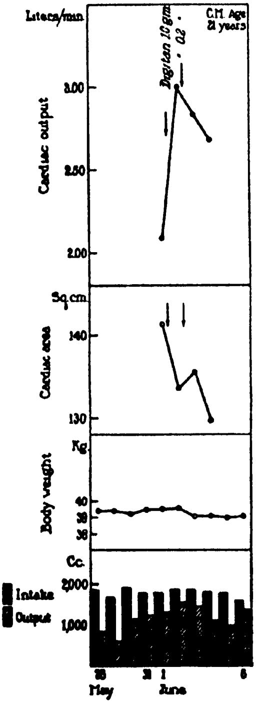


FIG. 14. In this figure is represented the effect of giving digitalis on cardiac output and cardiac size in C. M. (Case 4).

The rhythm of the heart was normal. Dyspnea was the only symptom of heart failure.

The cardiac output increased from 2.19 liters per minute to 3.00 liters, and cardiac size fell from 141.1 sq. cm. to 133.7 sq. cm. when digitan 1.0 gram was given (Table 3, Figure 14). Dyspnea disappeared. Although there was no evidence of accumulation of fluid, the output of urine increased slightly. The vital capacity increased. Alterations in the form of the T-waves of the electrocardiograms occurred.

Summary. In this patient who exhibited no signs of heart failure but whose cardiac output was nevertheless diminished, the administration of digitalis resulted in increase in cardiac output and diminished cardiac size.

In the second group are three patients suffering from auricular fibrillation who exhibited signs and symptoms of heart failure of the congestive type.

Case 5. S. G. (Hospital number 7902), a white man 31 years of age, was admitted to the hospital on November 9, 1931. He suffered from an attack of rheumatic fever at 26 years of age; involvement of the heart occurred at that time. Dyspnea and palpitation began 16 months before admission following over-exertion. He took digitalis irregularly. On admission the heart was enlarged. Auricular fibrillation was present. There were signs of mitral stenosis and insufficiency and of aortic insufficiency. Cyanosis was present. There were a few râles at the bases of the lungs posteriorly which disappeared within twenty-four hours. Edema of the extremities and ascites were not present; the liver was palpable. The *etiological* diagnosis was: rheumatic fever; the *anatomical*: cardiac hypertrophy, mitral stenosis and insufficiency, aortic insufficiency, left ventricular preponderance; the *physiological*: auricular fibrillation.

When admitted to the hospital he was under the influence of digitalis; the ventricular rate was slow, the pulse deficit small. On November 11, the cardiac output measured 2.58 liters per minute, the cardiac size, 154.9 sq. cm., and the vital capacity, 2200 cc. (Table 4, Figure 15). As the effect of digitalis wore off the ventricular rate became more rapid and the pulse deficit wider. Body weight increased and dyspnea became more severe. He complained of palpitation and cardiac pain. The liver became larger. On November 28, the cardiac output fell to 2.23 liters per minute, the size of the heart rose to 159.7 sq. cm. and the vital capacity fell to 1600 cc. On the administration of digitalis in the form of digitan 1.0 gram after measurements were made on November 28, diuresis occurred promptly, the ventricular rate became slower and the patient felt better. Twenty-four hours later his output increased to 3.40 liters per minute, the size of the heart fell to 153.6 sq. cm., and the vital capacity rose to 1800 cc. The administration of digitalis was continued until December 4. Diuresis continued, he lost weight and became free of the signs and symptoms of heart failure. As before, when the drug was no longer given, its effect wore off. Heart failure (cyanosis, dyspnea, palpitation, cardiac pain, enlargement of the liver, rapid ventricular rate, gain in body weight, decrease in vital capacity) reappeared. The cardiac output fell to 1.67 liters per minute (January 5) and the size of the heart increased to 164.6 sq. cm. Venous pressure measured 11.6 cm. of blood. Digitan 1.0 gram was given a second time. Diuresis again occurred promptly and the ventricular rate again became slow. The output increased to 2.47 liters per minute and the size of the heart fell

TABLE 4

Effect of Giving Digitalis on Cardiac Output, Cardiac Size, Vital Capacity, and Venous Pressure in Patients Exhibiting Auricular Fibrillation during Heart Failure

Case and hospital number		Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output	Cardiac area	Ventricular rate	Arterial pressure	Venous pressure	Vital capacity	Effect on T-wave of electro-cardiogram	Theo-calcin	Digitan	Time with reference to digitalis administration
	years	*1931*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*liters per sq.m. per minute*	*sq.cm.*	*minutes*	*mm. Hg*	*cm. blood*	*cc.*		*grams*	*grams*	
5 S. G. 7902 ♂	31	November 11*	61.0	165.5	1.68	242	84.0	2.58	1.50	154.9	64			2200	$-T_{12}$, $\mp T_3$			
		November 28	62.8		1.70	245	109.5	2.23	1.31	159.7	100			1600	T_{12} less neg., $+T_3$			Before
		November 28															1.0	
		November 29	62.9		1.70	255	75.0	3.40	2.00	153.6	68			1800	T_{12} more neg., $\mp T_3$			24 hours after
		November 30	62.4		1.68	240	95.9	2.50	1.50	156.5	68			1700				48 hours after
		December 1	61.4		1.68	245	91.9	2.67	1.59	157.7	65			1800			0.5 (Dec. 1–8)	
		December 3	59.3		1.66	213	89.7	2.37	1.43	155.4	67			1900				
		December 10	56.8		1.63	222	89.0	2.50	1.53	160.8	66			2200				
															Change in reverse direction			
		1932																
		January 5	58.4		1.64	218	130.7	1.67	1.02	164.6	100		11.6	1700	T_{12} less neg., $+T_3$			Before
		January 5															1.0	
		January 6	58.3		1.64	230	93.0	2.47	1.51	144.5	60		3.0	1950	T_{12} more neg., $\mp T_3$			24 hours after

TABLE 4—*Concluded*

Case and hospital number	Age	Date	Weight	Height	Body surface	Oxygen consumption	Arteriovenous oxygen difference	Cardiac output	Cardiac output	Cardiac area	Ventricular rate	Arterial pressure	Venous pressure	Vital capacity	Effect on T-wave of electrocardiogram	Theocalcin	Digitan	Time with reference to digitalis administration
	years	*1932*	*kgm.*	*cm.*	*sq. m.*	*cc. per minute*	*cc.*	*liters per minute*	*liters per sq.m. per minute*	*sq.cm.*	*minutes*	*mm. Hg*	*cm. blood*	*cc.*		*grams*	*grams*	
		January 7	57.2		1.63	212	83.7	2.53	1.55	161.3	53		4.9	2100				48 hours after
		January 9	55.9		1.62	214	86.9	2.46	1.52	160.3	60		4.0	2150			0.1	
		January 10															0.3	
		January 11	55.2		1.61	209	92.3	2.27	1.40	170.6	52		2.5	2300				
		1931																
6	51	May 20	59.0	163.0	1.63	199	81.5	2.44	1.50	185.3	91†	140/110		1650	$+T_1$, $\mp T_{23}$			Before
M. C.		May 20															1.0	
7489		May 21	58.1		1.62	208	74.6	2.79	1.72	170.5	56	160/110		1800	$\mp T_1$, $-T_{23}$			24 hours after
♀		May 22	56.6		1.61	214	62.0	3.45	2.14	167.3	60	130/110		1825	$\mp T_1$, $-T_{23}$			48 hours after
		May 27	56.4		1.60	212	66.0	3.21	1.99	167.4	82	130/110		1710	$\mp T_1$, $-T_{23}$			
		1932																
7	38	January 19	84.3	164.0	1.89	284	158.9	1.78	0.94	‡	108		9.5	1600	$+T_{123}$ *			Before
M. J.		January 19															0.8	
5783		January 20	83.1		1.88	275	110.8	2.48	1.32	‡	79		6.5	1600	$-T_{12}$, $\mp T_3$			24 hours after
♀		January 21														2.0		
		January 22														4.5		
		January 23	82.3		1.87	306	75.5	4.05	2.17	‡	81		3.5	1500		(daily)		
		January 30	77.0		1.82	295	89.2	3.31	1.82	‡	96		4.4	1700	$\mp T_1$, $+T_{23}$			

* Patient was under the influence of digitalis on admission to hospital.

† The radial pulse rate was counted in this patient.

‡ The size of the heart could not be measured; see text.

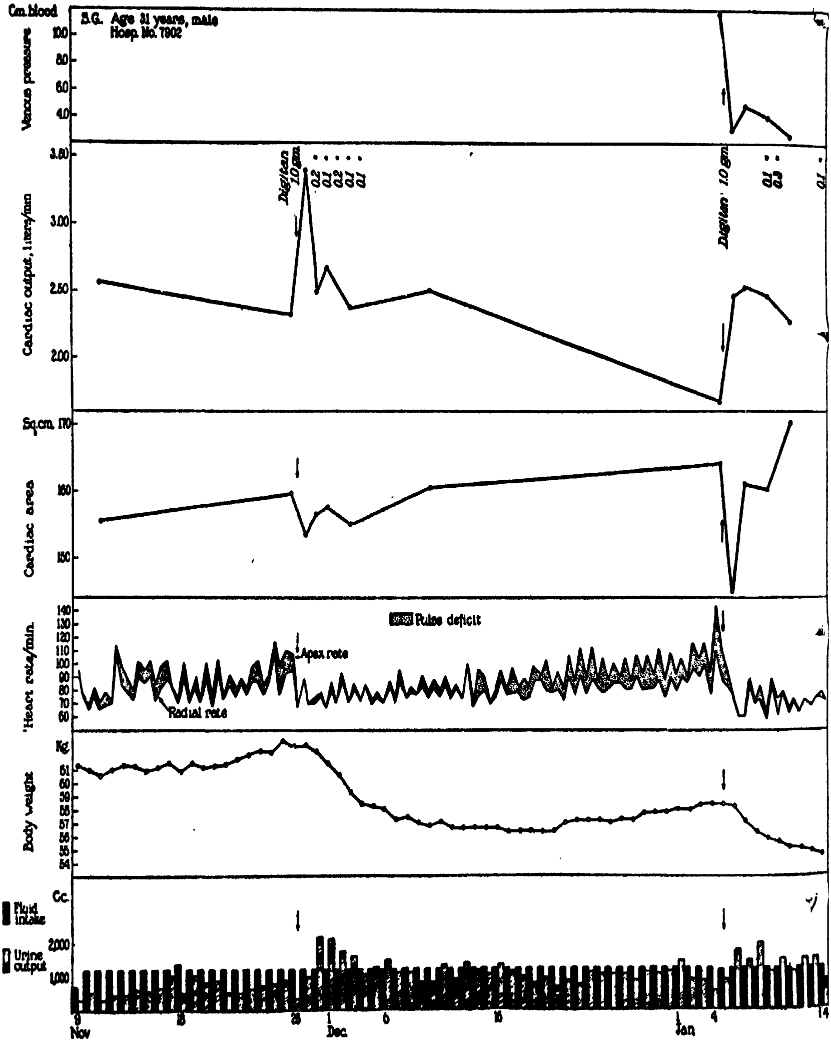


FIG. 15. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, and volume of urine in S. G. (Case 5), the subject of auricular fibrillation.

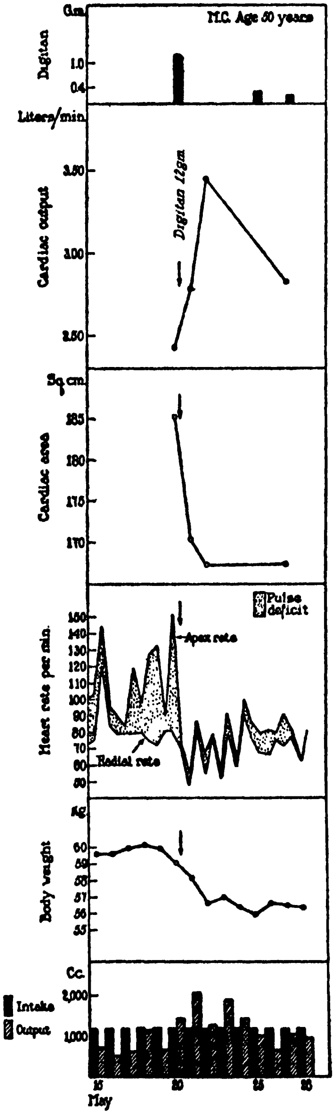


FIG. 16. In this figure is represented the effect of giving digitalis on cardiac output, cardiac size, and volume of urine in M. C. (Case 6), exhibiting auricular fibrillation.

to 144.5 sq. cm. The venous pressure fell to 3.0 cm. of blood and the vital capacity increased to 1900 cc. Diuresis continued until the patient became free of heart failure.

Summary. In this case the volume output was less and the size greater during heart failure than during the state of compensation. On administration of digitalis, cardiac output increased, cardiac size diminished, vital capacity increased, venous pressure decreased, ventricular rate became slower, and the patient became free of the signs and symptoms of heart failure. As the effect of digitalis wore off, changes occurred in the reverse direction.

Case 6. M. C. (Hospital number 7489), a woman 51 years of age was admitted to the hospital on May 6, 1931. Dyspnea appeared first when she was 46 years of age. A first attack of heart failure occurred two years later. On admission she was suffering from a fourth attack of heart failure. She had been complaining of shortness of breath and swelling of the feet and abdomen for four weeks. The *etiological* diagnosis was: unknown; the *anatomical*: enlargement of heart, chronic myocarditis, right ventricular preponderance, mitral insufficiency; the *physiological*: auricular fibrillation, ventricular premature contractions, heart failure of the congestive type (fourth attack).

The cardiac output was 2.44 liters per minute on May 20, and the size of the heart 185.3 sq. cm. (Table 4, Figure 16). She had not taken digitalis since admission to the hospital. The ventricular rate was rapid, the pulse deficit was great, there were a few râles at the bases of both lungs posteriorly; there was edema of the extremities; ascites was present, the liver was enlarged. On the same day she was given digitalis (digitan 1.0 gram). The ventricular rate became slower, diuresis occurred, she lost weight, râles, edema and ascites disappeared, and the liver became smaller. Two days later (May 22) the cardiac output rose to 3.45 liters per minute and the size of the heart fell to 167.3 sq. cm. The vital capacity rose from 1650 to 1825 cc.

Summary. In this patient exhibiting auricular fibrillation, the cardiac output was diminished and the size of the heart large during heart failure. On the administration of digitalis the output rose, the size diminished, and the patient became free of edema.

Case 7. M. J. (Hospital number 5783), a white woman, 38 years of age, was admitted to the hospital on January 11, 1932. Hypertension had been present for five years and auricular fibrillation for three years. On admission, suffering from a seventh attack of heart failure, she exhibited marked edema of the extremities and enlargement of the liver. There were a few râles at the bases of the lungs posteriorly. The heart was large, the ventricular rate rapid. The systolic blood pres-

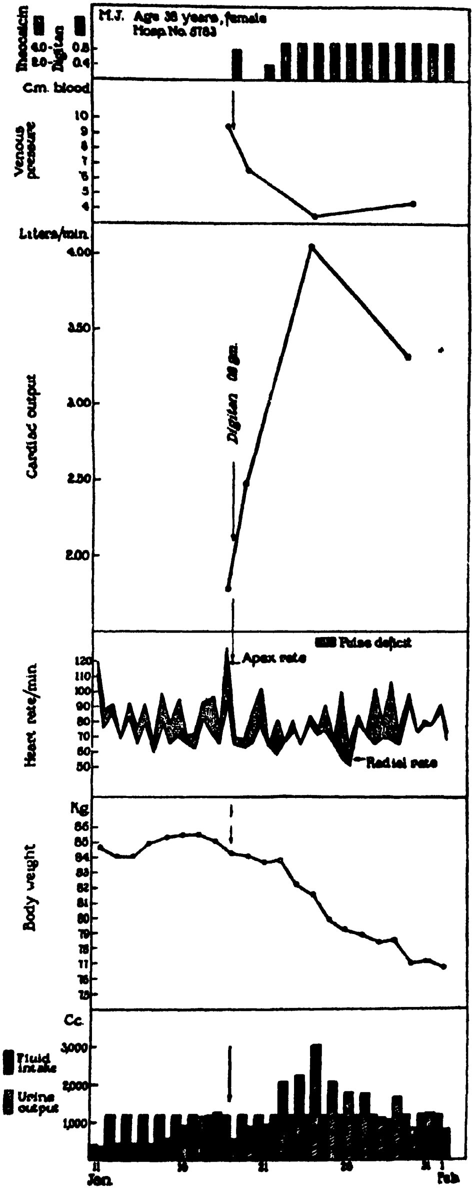


FIG. 17. In this figure is shown the effect of giving digitalis and theocalcin on cardiac output, cardiac size, and output of urine in M. J. (Case 7), the subject of auricular fibrillation.

sure measured 180 mm. Hg, the diastolic, 140 mm. Hg. The *etiological* diagnosis was: arterial hypertension; the *anatomical*: enlargement of heart, left ventricular preponderance; the *physiological*: auricular fibrillation, intraventricular heart-block, heart failure of the congestive type.

The cardiac output measured 1.78 liters per minute on January 19, and the venous pressure, 9.5 cm. blood (Table 4, Figure 17). The heart was enlarged to such an extent that its left border, in the x-ray photographs, could not be distinguished from the shadow of the chest wall; for this reason its size was not measured. Digitalis (digitan 0.8 gram) was given; the ventricular rate decreased and 24 hours later (January 20) the cardiac output rose to 2.48 liters per minute, and the venous pressure fell to 6.5 cm. blood. As diuresis did not occur at once, the administration of theocalcin was begun on January 21. Cardiac output increased to 4.05 liters per minute and the venous pressure fell to 3.5 cm. of blood. Diuresis occurred and persisted until the patient became free of edema.

Summary. In this patient exhibiting auricular fibrillation with a rapid ventricular rate, the cardiac output was diminished during heart failure. Following the administration of digitalis, output increased, venous pressure fell, and ventricular rate became slower. The administration of theocalcin was more effective than digitalis in the relief of heart failure. It resulted also in greater increase in cardiac output.

SUMMARY

The experience gained from a study of these patients makes it clear that (1) the volume output of blood per minute from the heart in human beings is diminished during heart failure, both when the cardiac mechanism is normal and when auricular fibrillation is present.

(2) During heart failure the size of the heart is larger than it is when in a state of compensation.

(3) When improvement results, the administration of digitalis occasions: (*a*) *increase* in cardiac output; (*b*) *decrease* in cardiac size; (*c*) *decrease* in venous pressure; (*d*) increase in vital capacity; (*e*) slowing of the ventricular heart rate. (*f*) Changes in the form of the electrocardiogram are observed at this time.

(4) The administration of theocalcin was more effective in the relief of heart failure than digitalis in two patients. Giving it occasioned (*a*) increase in cardiac output, greater in amount than resulted from giving digitalis; (*b*) decrease in cardiac size; and (*c*) decrease in venous pressure.

DISCUSSION

In all subjects, normal as well as those suffering from heart failure, alterations in form of the T-waves of the electrocardiograms followed the administration of digitalis. They were frequently the earliest effects observed. The alterations were not uniform; the most characteristic and usual one was negativity of T_3 (21); in certain cases T_1 and T_2, however, also became negative; T_1 or T_2 or T_3 sometimes became diphasic. On the other hand, waves which were negative became positive, and finally changes in amplitude only, occurred. The point to be emphasized is that *change* in form of the waves *always* took place.

There is ample experience with theocalcin in the treatment of heart failure to show that it is often useful in the relief of edema when digitalis fails (22). The new experience, although limited to observations in two patients, supplies evidence that in addition its action is accompanied by increase in cardiac output and decrease in cardiac size and in venous pressure. What the mechanism is by which theocalcin accomplishes these results is still unknown. Various possibilities suggest themselves, a xanthin effect on the rate of coronary flow, a calcium ion effect on heart muscle, or still another form of calcium action.

It has already been shown in Part I of this paper that the results of the administration of digitalis to normal individuals differ in several respects from those just described in patients. These differences include: (*a*) *decrease* in cardiac output; (*b*) *decrease* in cardiac size; (*c*) no significant alteration in the level of venous blood pressure; (*d*) no change in vital capacity.

A matter of major importance which has emerged from conducting these researches is the paradox that one and the same drug, digitalis, when given to human beings has, in normal persons, one effect, and in persons the subjects of heart disease, another; decrease in the output of blood in the former, increase in the latter, as Burwell, Neighbors, and Regen found (1). In parallel situations in dogs (normal (23) and pathological (24)) the results resemble those now reported. Is the difference in result due to an essential difference in action or is it due to a difference in the essential conditions which are being investigated?

In both normal and diseased hearts, in human beings and in dogs the action of digitalis has, at least in one respect, an identical consequence; in both, the size of the heart becomes smaller. It has also another consequence; the vigor of its action increases. If this consequence were not known from former experience (Cohn and Stewart (24, 25, 26)) it would have become apparent from the present ones in which the output of blood in diseased persons increases. The difference in the effect in the two types of heart, normal and pathological, results, so we think and as we have formerly stated, from the fact that normal hearts when under its influence become smaller but, being smaller pumps, expel smaller volumes, though contraction is more effective. The net result of its action, if it depends upon an effect on the hepatic veins, does so to a minor, perhaps to a negligible, degree only. Pathologically enlarged hearts likewise become smaller, but not so small that their cavities shrink to dimensions less than normal; in point of fact, though they decrease, their final dimensions are larger perhaps than when they were in their healthy state. They attain, due to the action of digitalis, sizes commensurate with ejecting larger volumes than were possible in the state of failure. Whether decrease in size to the extent to which it occurs facilitates the result, the expulsion, namely in heart failure, of larger quantities than before; or whether the increase in contractile capability brings it about, the fact is nevertheless clear that the heart has become smaller and that the circulation which was insufficient before, has become sufficient. The evidence for improvement is to be found in the amelioration of the symptoms and in changes in the other physical signs.

The explanation of these events may perhaps be found in Starling's theory dealing with the length of the muscle fibers of the heart (27). The theory is too well known to require repetition. It is not a wide stretch to believe that it is applicable in these cases in which there is such clear evidence of change in size of the heart and of increase in its functional capacity.

There is required still, consideration of that aspect of the problem which Dock and Tainter (3, 4) have brought forward. The major part of the argument which deals with their contention and the evidence which leads us to take a different view, is contained in a former paper (28). It is necessary to discuss one more phase of the matter. In cases where the output becomes smaller, as in the normal heart, the point has been made (3, 4) that this is due, not to a change in diastolic size of the heart, a change in tone, but to decrease in inflow, a result of constriction of the hepatic veins, indicated by the fact that the venous pressure is low. Low venous pressure is, on the latter assumption, regarded as the sign of a small volume of blood available for inflow into the heart. Low venous pressure was found by Dock and Tainter in dogs, which they studied, for a maximum of two hours after the injection of a digitalis substance.

The plain result of the measurement of the venous pressure in normal human beings is that a change in pressure parallel with the volume of the output does not occur. Based on these observations, and in view of the anatomical arrangement in the liver of human beings, the mechanism proposed by Dock and Tainter seems untenable. A reason for the difference in interpretation can be given to the last statement, depending on a difference in the times when their observations and ours were made—theirs after an interval varying from a few minutes to two hours, ours after many hours and as a matter of fact after several days. The duration of their observations was, as has been pointed out, brief. If the course of the entire subsequent action in dogs is determined by events in these early minutes, we have no counter argument to offer. But it seems unlikely in healthy human beings that, if the outflow is small and the venous pressure normal or even elevated, as in several instances, the level of the venous pressure can be responsible for the result, in the sense in which Dock and Tainter wish to attribute to it a deciding influence. The venous pressure, far from being low, is too inconstant in its behavior to bear the burden of responsibility for so conspicuous an action as the decrease in output. What the venous pressure is in healthy intact dogs, after hours or days, is still unknown. Information on this point is desirable.

On the other hand we have demonstrated again, and it is an observation which is now well known, that fall in venous pressure is not an infrequent occurrence following the administration of digitalis to patients suffering from heart failure and edema. *Increase* in cardiac output occurs in such cases, as these observations show, at a time when venous pressure falls; venous pressure, in short, is high when the output is small and falls as output increases. The venous pressure is high under these circumstances not because the venous return is great but because the heart is incapable of propelling through its cavities all the blood which comes to it.

It seems necessary, therefore, in the case of normal hearts, the evidence being what it is, to hold belief in the theory that decrease in outflow is due to increase, expressed as decrease beyond the natural, perhaps optimal length, in tone of the heart muscle.

CONCLUSIONS

1. A consequence of the action of digitalis is to decrease the volume output of blood per minute from the heart in normal human beings and to decrease its size.
2. The volume output of blood per minute from the heart which is in failure is diminished and its size larger than when it is in a state of compensation.
3. Following the administration of theocalcin in patients during heart failure, cardiac output increases and cardiac size and venous pressure diminish.
4. Giving digitalis increases the volume output of blood per minute from failing hearts and decreases their size.
5. Digitalis, we think, has similar, perhaps identical, actions both in normal and in diseased hearts; it *decreases* cardiac size and *increases* the extent of ventricular contraction (23). The consequence of these actions is that the volume of the cardiac output which results differs, depending on an initial difference in size of the ventricular cavities, in the two situations. In the one, the normal heart, it becomes too small, in the other, the diseased heart, it develops a suitable size.

BIBLIOGRAPHY

1. Burwell, C. S., Neighbors, De W., and Regen, E. M., J. Clin. Invest., 1928, v, 125. The Effect of Digitalis upon the Output of the Heart in Normal Man.
2. Harrison, T. R., and Leonard, B. W., J. Clin. Invest., 1926, iii, 1. The Effect of Digitalis on the Cardiac Output of Dogs and Its Bearing on the Action of the Drug in Heart Disease.
3. Dock, W., and Tainter, M. L., J. Clin. Invest., 1929–30, viii, 467. The Circulatory Changes after Full Therapeutic Doses of Digitalis, with a Critical Discussion of Views on Cardiac Output.

4. Tainter, M. L., and Dock, W., J. Clin. Invest., 1929–30, viii, 485. Further Observations on the Circulatory Actions of Digitalis and Strophanthus with Special Reference to the Liver and Comparisons with Histamine and Epinephrine.
5. Field, H., Bock, A. V., Gildea, E. F., and Lathrop, F. L., J. Clin. Invest., 1924, i, 65. The Rate of the Circulation of the Blood in Normal Resting Individuals.
6. Weiss, S., and Ellis, L. B., J. Clin. Invest., 1929–30, viii, 435. Circulatory Measurements in Patients with Rheumatic Heart Disease Before and After the Administration of Digitalis.
7. Henderson, Y., and Haggard, H. W., Am. J. Physiol., 1925, lxxiii, 193. The Circulation and Its Measurement.
8. Ringer, M., and Altschule, M., Am. Heart J., 1929–30, v, 305. Studies on the Circulation. II. Cardiac Output in Diseases of the Heart and Under the Influence of Digitalis Therapy.
9. Grollman, A., Am. J. Physiol., 1929, lxxxviii, 432. The Determination of the Cardiac Output of Man by the Use of Acetylene.
10. Baumann, H., and Grollman, A., Ztschr. f. klin. Med., 1931, cxv, 41. Über die theoretischen und praktischen Grundlagen und die klinische Zuverlässigkeit der Acetylenemethode zur Bestimmung des Minutenvolumens.
11. Grollman, A., Proger, S., and Dennig, H., Arch. f. exp. Path. u. Pharm., 1931, clxii, 463. Zur Bestimmung des Minutenvolumens mit der Azetylenemethode bei Arbeit, bei normalen und kranken Menschen.
12. Grollman, A., Am. J. Physiol., 1929, xc, 210. Physiological Variations in the Cardiac Output of Man. VI. The Value of the Cardiac Output of the Normal Individual in the Basal, Resting Condition.
13. Levy, R. L., Arch. Int. Med., 1923, xxxii, 359. The Size of the Heart in Pneumonia. A Teleroentgenographic Study, with Observations on the Effect of Digitalis Therapy.
14. Grollman, A., Am. J. Physiol., 1929, lxxxix, 366. Physiological Variations in the Cardiac Output of Man. III. The Effect of the Ingestion of Food on the Cardiac Output, Pulse Rate, Blood Pressure, and Oxygen Consumption of Man.
15. Soderstrom, G. F., Barr, D. P., and Du Bois, E. F., Arch. Int. Med., 1918, xxi, 613. Clinical Calorimetry. XXVI. The Effect of a Small Breakfast on Heat Production.
16. Grollman, A., Am. J. Physiol., 1929, lxxxix, 157. Physiological Variations in the Cardiac Output of Man. II. Changes in the Cardiac Output, Metabolism, Blood Pressure, and Pulse Rate of Man Following the Ingestion of Fluids.
17. Steele, J. M., Effect of Digitalis on Body Temperature. (To be published.)
18. Taylor, F. A., Thomas, A. B., and Schleiter, H. G., Proc. Soc. Exp. Biol. and Med., 1930, xxvii, 867. A Direct Method for the Estimation of Venous Blood Pressure.

19. Keefer, C. S., and Resnik, W. H., Arch. Int. Med., 1928, xli, 769. Angina Pectoris; a Syndrome Caused by Anoxemia of the Myocardium.
20. Grollman, A., Am. J. Physiol., 1928, lxxxvi, 285. The Effect of Variation in Posture on the Output of the Human Heart.
21. Cohn, A. E., Fraser, F. R., and Jamieson, R. A., J. Exp. Med., 1915, xxi, 593. The Influence of Digitalis on the T-wave of the Human Electrocardiogram.
22. Stewart, H. J., J. Clin. Invest., 1930, viii, 389. The Use of Theocalcin in the Treatment of Heart Failure of the Congestive Type.
23. Stewart, H. J., and Cohn, A. E., J. Clin. Invest., 1932, xi, 897. Studies on the Effect of the Action of Digitalis on the Output of Blood from the Heart. II. The Effect on the Output of the Hearts of Dogs Subject to Artificial Auricular Fibrillation.
24. Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1928, vi, 53. The Relation Between Cardiac Size and Cardiac Output per Minute Following the Administration of Digitalis in Normal Dogs.
25. Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1928, vi, 79. The Relation Between Cardiac Size and Cardiac Output per Minute Following the Administration of Digitalis in Dogs in Which the Heart is Enlarged.
26. Cohn, A. E., and Stewart, H. J., J. Clin. Invest., 1924, i, 97. Evidence that Digitalis Influences Contraction of the Heart in Man.
27. Starling, E. H. The Linacre Lecture on the Law of the Heart, given at Cambridge, 1915. Longmans, Green and Co., London, 1918.
28. Cohn, A. E., and Steele, J. M., J. Clin. Invest., 1932, xi, 871. Studies on the Effect of the Action of Digitalis on the Output of Blood from the Heart. I. The Effect on the Output of the Dog's Heart in Heart-Lung Preparations.

[Reprinted from THE AMERICAN JOURNAL OF PHYSIOLOGY, October, 1932, Vol. 102, No. 1, pp. 167–182]

STUDIES OF THE INFLUENCE OF PULMONARY MOTION AND DISTENTION ON MOVEMENTS OF THE THORAX

I. CHEMICAL AND NERVOUS FACTORS INVOLVED IN ESTABLISHING APNEA

II. THE RELATION BETWEEN PULMONARY AND THORACIC MOVEMENTS

BY ALBERT J. ANTHONY, ALFRED E. COHN, AND J. MURRAY STEELE, JR.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 24, 1932)

PART I. CHEMICAL AND NERVOUS FACTORS INVOLVED IN ESTABLISHING APNEA. Pulmonary ventilation is adapted to the varying needs of the organism by several different regulatory mechanisms. The factors acting on the respiratory center through the circulating blood, so-called chemical regulation, have been studied by physiologists and clinicians during the last decade, while the problems of nervous regulation have been somewhat neglected.

Physiologists, and in particular clinicians, until recently have been satisfied with the Hering-Breuer (1) theory of auto-regulation. According to this conception, the respiratory movements are regulated through the vagus nerves, each distention of the lungs occasioning a centripetal impulse resulting in an expiration, and each collapse a centripetal one resulting in an inspiration. In the physiological literature several experimental observations have appeared which are, however, opposed to this theory and which suggest a different and more complicated nervous control.

Neither chemical nor nervous factors alone regulate respiration; for, as Haldane (2) states, "We cannot separate the nervous from the chemical control of breathing, since each determines the other at every point." It is, therefore, only in combination with chemical factors that the nervous influences can be adequately investigated. For this study we have selected a relatively simple problem, so-called vagal apnea.

It has been frequently demonstrated (Lockenberg (3), Guttmann (4), Head (5), Christiansen and Haldane (6)) 1, that a period of apnea following over-ventilation is prolonged by distention and terminated or shortened by collapse of the lungs; 2, that during normal ventilation, pulmonary distention produces a period of apnea; and 3, that distention of the lungs retards the respiratory rate. These phenomena do not occur after section of both vagi. Because distention of the lungs with nitrogen results in a period of apnea, just as when the lungs are distended with air, it has been thought that a purely nervous mechanism is implicated. This conception was held at a time, however, when oxygen was still considered to be the most important gas in the mechanism of respiration. Haldane and his collaborators have demonstrated that apnea produced by distention can be prolonged by previous over-ventilation, and shortened by administration of carbon dioxide, and have shown, therefore, that so-called vagal apnea can be influenced by chemical factors. They hold the view that this is not true apnea, but markedly prolonged expiration, and "that the term vagus apnea is an entire misnomer."

In order to learn how great an influence each of these factors, chemical and nervous, has on apnea resulting from distention, it was necessary to choose a definite and readily manifest point or level in chemical regulation. This level is probably represented by the threshold value of the respiratory center, which we define as the level to which the strength of the stimulus in the blood must rise during apnea induced by over-ventilation, before a respiratory movement occurs. The use of this level for this purpose involves the same principle used by Filehne (7) and Knoll (8), and later by Wieland (9), to ascertain the degree of irritability of the respiratory center.

Since the threshold stimulus is not necessarily identical with the usual respiratory stimulus, it was necessary to learn if, and how far, the threshold value of the respiratory center is influenced by pulmonary distention and collapse. To this end a series of samples of blood was taken for analysis during the latter part of, and at the end of, a period of artificially induced apnea.

EXPERIMENTAL PROCEDURE. The following experimental procedure was employed. Only dogs were used. The animals were anesthetized with chloralose injected intravenously in a dosage of 0.1 gram per kilogram of body weight, supplemented occasionally with small amounts of the drug if they became restless. A tracheal tube was inserted. The natural minute volume of respired air was measured. Later, artificial respiration was maintained by means of a Starling pump, the stroke volume and rate of which were so adjusted as to give approximately the desired respiratory minute volume. Bilateral pneumothorax was then created. At first this was accomplished by removing at least 6 cm. of two middle ribs on each side. Later, the sternum was split from above downward to the point at which the 6th or 7th costal cartilage was attached. Incisions from the lower end of this opening were then made by cutting laterally along the borders of the 7th ribs. The flaps so made were fastened back. When thoracic movements occurred, the lower intact portion of the thorax was found to move freely and independently of the motion of the lungs. A Y-shaped glass cannula was so introduced into a lateral branch of the deep femoral artery that the opening lay almost in the main artery. By this device the flow of arterial blood past the mouth of the cannula was free and uninterrupted. Heparin was then added to the dog's blood and the cannula closed with rubber tubes and clamps.

The ventilation was varied by appropriate adjustments in the stroke and rate of the Starling pump in order to bring about both over- and under-ventilation. Distention of the lungs was effected in one of two ways: 1, when sudden distention during a period of apnea was desired, room air was introduced into the lungs of the animal from an air-tight 3-liter glass jar by suddenly distending a rubber bag contained within it; 2, if distention was to be maintained during ventilation, expiratory resistance was increased by submerging the expiratory tube in water to a desired depth. A three-way stopcock inserted into the inspiratory tube permitted collapse of the lungs or the omission of ventilation during one or more strokes of the pump.

The records were made on smoked paper by writing levers. Intra-tracheal pressure was recorded by a Marey capsule connected by a side opening with the intra-tracheal tube. Abdominal respiration was recorded by transmitting changes of pressure in a rubber bag held snugly to the abdomen by a leather cuff to the lever of a Marey capsule. The movements of the lower thorax were transmitted by thread over pulleys to a rod supporting a recording lever and moving in a vertical bearing. The arrival of the piston of the pump making and breaking contact at the end of the expiratory stroke was recorded by a signal magnet.

A number of samples of blood were drawn in rapid succession from the femoral cannula and deposited in test tubes under liquid paraffin by means of a special rotating stopcock.[1] Carbon dioxide and oxygen of the blood were analyzed according to the method of Van Slyke and Neill (10). The pH of the blood was measured by the colorimetric method of Hastings and Sendroy (11).

The volume of air supplied by the pump was regarded as enough to bring about either over- or under-ventilation depending upon whether it was greater or less than the volume breathed spontaneously by each dog after the introduction of the tracheal cannula. Over- or under-ventilation having been attained, artificial ventilation was stopped. The lungs were then either allowed to collapse by removing the expiratory resistance, or were distended with room air by the method just described. A series of samples of blood was taken toward the end of the period of apnea. To avoid differences in the general state of the animals, the time between the taking of the samples was as short as possible (15 minutes). Because over- and under-ventilation are responsible for marked disturbances in the acid base equilibrium, the periods of ventilation preceding the taking of samples were equal in duration.

The question at once arises: is the sample of blood taken from the femoral artery identical in composition with that arriving at the respiratory center at the same moment? Identity would, it seems, depend on whether the two points are equidistant from the heart, and on whether the velocity in the two arteries is the same. The distance to the femoral artery is obviously greater than that to the respiratory center, but the difference may be compensated in whole or in part by decreased velocity in the precapillary vessels supplying the center The discrepancy in time cannot be more than 3 or 4 seconds and need not be considered in this connection since the same difference exists both in the state of distention and in that of collapse.

---

[1] This stopcock was made of glass and first used in January, 1931. Blood entered through an opening in the bottom of the center plug emerging on the side. Eight perforations through the jacket in a circle at the level of the opening in the center plug lead, by rubber connections, to openings into the lower ends of the glass sample tubes. The sample tubes are held by a stout rubber band against the outer surface of the jacket. The whole apparatus is then filled with paraffin oil. By revolving the jacket with the sample tubes fixed to it, the tubes can be successively and rapidly connected to the arterial needle or cannula.

RESULTS. The direct influences of distention and collapse of the lungs on the respiration have already been described. The term distention, or degree of distention, is used to designate only the static factor; that is to say, the state of being distended in distinction to the state of being collapsed. The use of the term is intended to exclude any dynamic factors as far as the lungs are concerned.

When artificial ventilation was stopped with the lungs in *collapse*, thoracic movements began in 14 seconds, with a frequency of 30 per minute (fig. 1a). As soon as artificial respiration was again instituted, the depth and frequency of the thoracic movements decreased. But when artificial respiration was stopped with the lungs *distended* (fig. 1b), apnea lasted 88 seconds and thoracic movements began with a frequency of only 12 per minute. If an animal was slightly *under*-ventilated, it exhibited regular thoracic movements of its own. In collapse of the lungs (fig. 2a) no apnea resulted; the frequency was 24 per minute. In distention (fig. 2b) there was apnea for 15 seconds; thoracic movements then began with a frequency of 10 per minute. After bilateral vagotomy, collapse and distention of the lungs had no influence on the type of thoracic motion (fig. 3). That the dog under description was over-ventilated is shown by the occurrence of apnea before vagotomy; after vagotomy, this phenomenon did not recur. These experiments establish the fact that distention can occasion apnea, or prolong it, if it exists already, and can likewise decrease the frequency of thoracic motion, but only if the vagi are intact. It is also a fact that the length of an apneic period, occasioned by distention of the lungs, can be influenced by the degree of previous ventilation.

The results of the chemical analyses of the blood in all of the experiments show changes in the same direction. Three examples, therefore, suffice.

At the beginning of a period of apnea due to over-ventilation, the oxygen saturation is nearly normal, while the carbon dioxide content is at the lower limit of normal (fig. 4). If ventilation is stopped when the lungs are in collapse, the concentration of oxygen decreases and that of carbon dioxide increases, rapidly; when they are distended these changes go on much more slowly. When the lungs are in collapse the first thoracic movement takes place at a much higher oxygen, and much lower carbon dioxide concentration than when they are distended.

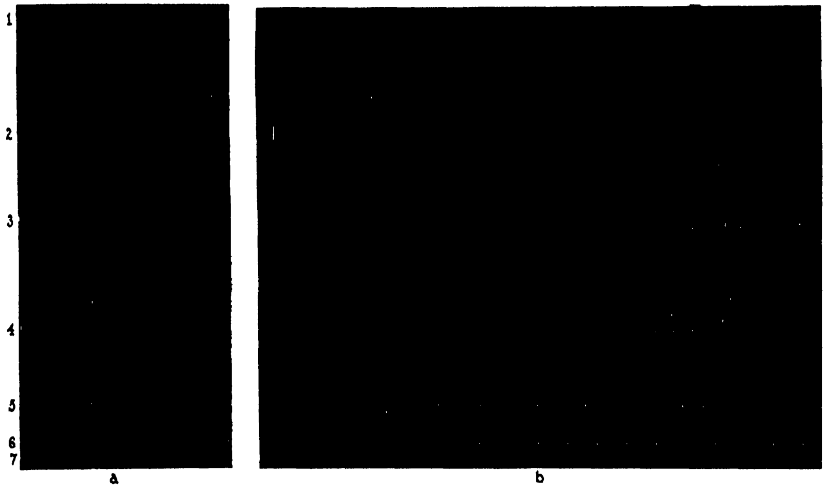


FIG. 1. The effect is shown upon the duration of apnea in a dog which has been the subject of *over*-ventilation when ventilation was arrested *a*, in the state of collapse, and *b*, in a state of distention of the lungs. The curves from above downward are made by:

1. A signal magnet in circuit with the respiratory pump. At the end of expiration, the shaft of the piston made contact.

2. A tambour connected by tubing with the tracheal cannula. Upstrokes of the curve indicate inspiration.

3. A lever moving vertically, connected by string with the lower portion of the thorax. Upstrokes represent inspiration.

4. A tambour connected by tubing with inflated rubber bags applied to the abdomen, to record motions of the abdominal wall. Upstrokes represent inspiration.

5. A signal magnet, to record the time when maneuvers were carried out; as when samples of blood were taken.

6. A time signal every 10 seconds.

7. A time signal every 1 second.

The curves in figures 2, 3, 8, 9, 10, 11 record similar events.

Although the concentration of carbon dioxide in the blood of the dog now being described was low, an indication of over-ventilation, apnea did not occur until the lungs were distended during the second part of the experiment (fig. 5). This result might have been due to low concentration of oxygen due in turn to changes in the lungs, the result probably of beginning pulmonary edema. Although pathological conditions were present in this experiment, the various changes in concentration of the gases in the blood at the onset of the animal's

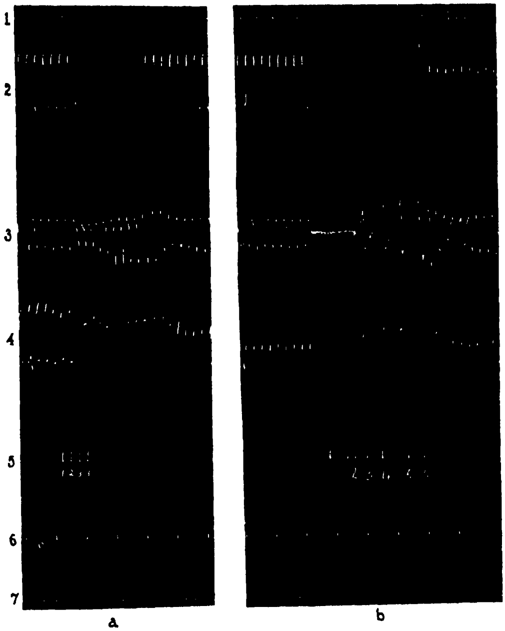


FIG. 2

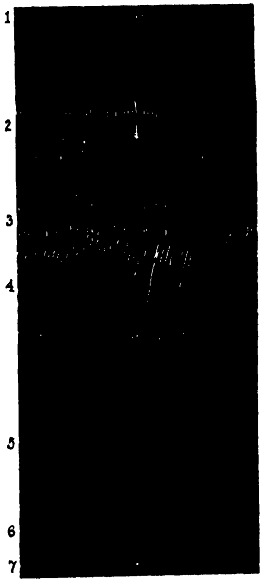


FIG. 3

FIG. 2. The effect is shown upon the duration of apnea in a dog which has been the subject of *under*-ventilation both when ventilation was arrested *a*, in a state of collapse, and *b*, in a state of distention of the lungs. The curves record events similar to those in figure 1.

FIG. 3. The effect is shown of bilateral vagotomy in a dog which has been the subject of *over*-ventilation, when ventilation was arrested, the lungs being in a state of collapse (portion of the curve to the left of the ordinate) and in a state of distention. The curves represent events similar to those in figure 1.

thoracic movements, after collapse and after distention of the lungs, were in the same direction as in the first experiment described. Since

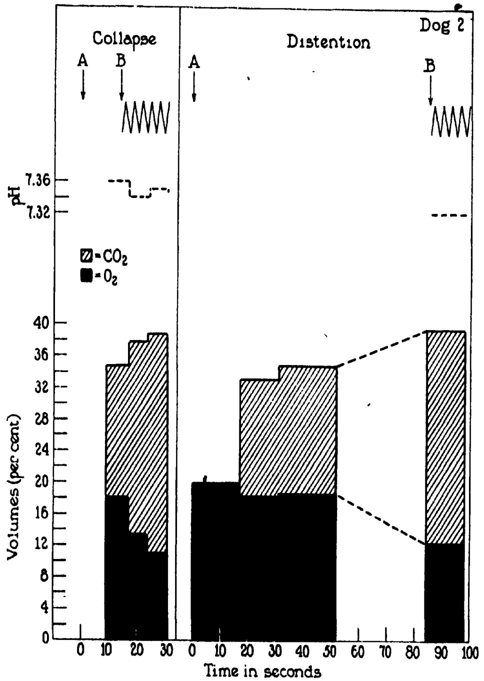


FIG. 4

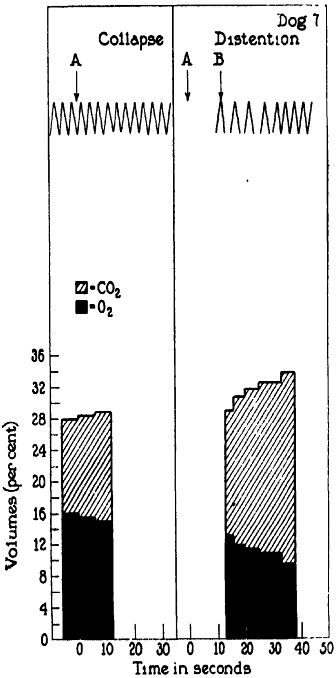


FIG. 5

FIG. 4. Chart of the relation of the concentrations of carbon dioxide and oxygen in the blood of a dog previously over-ventilated by artificial means to the onset of thoracic movements following discontinuance of artificial ventilation with 1, collapse, and 2, distention of the lungs. Dog 2, the same one from which the tracings in figure 1 are taken is the subject of this chart. In this figure and in figures 5 and 6 the arrows at *a* indicate the moment at which artificial ventilation was stopped, those at *b* the moment at which thoracic movements began. The line immediately beneath the arrows shows the periods during which thoracic movements occurred. Apnea was otherwise present. Hydrogen ion concentration of the blood is shown in broken lines.

FIG. 5. Chart showing the same relations as those described in figure 4, but with the dog under- instead of over-ventilated. The tracings in figure 2 are taken from the same animal (dog 7). It is to be noted that no apnea occurred when the lungs were collapsed.

a period of apnea did not occur during collapse, the threshold value was not obtained. The recorded value for the level of the stimulus of the blood was, of course, greater than the true threshold value. The difference, therefore, between the threshold stimulus in distention and collapse is really greater than that shown.

Somewhat different results are exhibited in figure 6, obtained from an animal in which the saturation of oxygen at the beginning of the apneic period was nearly normal. Since there had been over-ventilation, the content of carbon dioxide was low. While the period of apnea lasted only 3 seconds when the lungs were in a state of collapse, the duration when distended was 77 seconds. It is apparent from these three experiments that the changes in the gases of the blood progress more slowly when the lungs are distended than when they are in collapse.

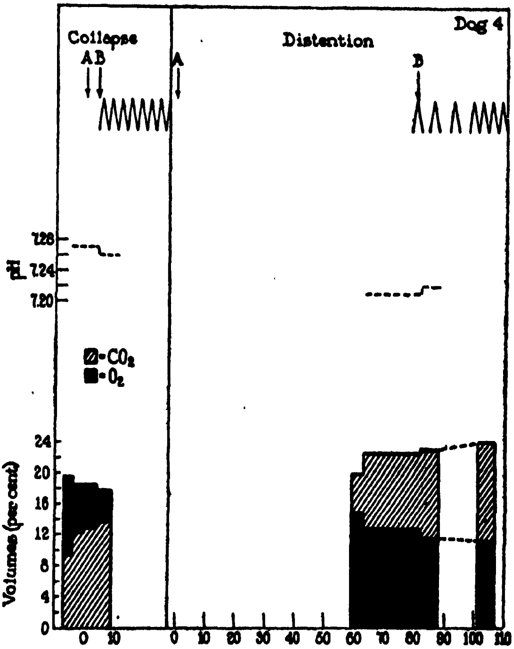


FIG. 6. Chart showing the same relations as described in figure 4 in a markedly over-ventilated animal.

Before passing on to a discussion of these results it is to be noted that in all cases the apnea which occurred during distention was true apnea and not merely prolonged expiration as described by Haldane.

Discussion. If the first thoracic movement marking the end of apnea denotes the moment at which the strength of the stimulus in the blood reaches the threshold value required to actuate the respiratory center, it is apparent, as the result of these experiments, that this value is greater in distention than in collapse of the lungs. The mechanism responsible for this difference may, however, be very complicated. For instance, the medullary centers for the thoracic muscles may change their sensitivity to the impulses coming from the respiratory center. We can only say, therefore, that distention of the lungs has the same effect as decrease in irritability of the respiratory center. Furthermore, following apnea when the lungs were in collapse, thoracic movements began at a much more rapid rate than when they were distended, which fact strongly suggests that the degree of distention of the lungs has an influence also on the character of thoracic motion.

We can confirm the fact that after bilateral vagotomy, distention of the lungs does not occasion apnea, nor does distention or collapse any longer influence the rate of the thoracic movements. From vagotomy experiments such as these, many authors have concluded that the centripetal impulses produced by distention and by collapse of the lungs run in the vagus. This conclusion is not justified as long as other nervous communications exist (as in the Plexus vagi).

The appraisal of this form of experiment is difficult and requires great caution because the removal of vagal influence changes the sensitivity of the respiratory center to other stimuli such as that of carbon dioxide. It might, for instance, become less sensitive to impulses coming to it through other nervous pathways. This possibility has already been discussed by Breuer. He says "One could suppose that the peripheral stimulus produced by lung distention acts on the medulla as before, and therefore does not go by way of the vagus; but the respiratory center has become too insensitive, too insufficiently labile, for stimuli to produce marked changes in respiratory rhythm." Breuer thinks that this theory is unlikely, although no experiments have been performed which exclude the possibility of such a mechanism. It is therefore necessary for the present to conclude no more than that the degree of distention of the lungs has an effect on respiration only when the vagi are intact.

There remains for discussion the question as to how the centripetal impulses due to distention of the lungs arise. That intra-thoracic pressure is of no importance, as Breuer has already shown, becomes apparent from the fact that no changes occurred in our experiments since the thoracic cavity was in open communication with the atmosphere. It is more difficult to decide whether distention of the pulmonary tissue itself or change in intra-alveolar pressure acts as the stimulus, since these two, being interdependent to a marked degree, can probably not be separated. Since only the difference between intra- and extra-alveolar pressure can be effective, to test the influence of respiration under negative pressure is of no avail. The possibility that centripetal impulses result primarily through the influence of distention of the lungs on the extra-pulmonary circulatory apparatus, complicates the problem; the reference to this possibility is due to the fact that in recent investigations it has been learned that changes in blood pressure influence the respiration (12), (13). Heymans has shown that a rise in blood pressure in the carotid sinus may be accompanied by the occurrence of apnea. In the course of these experiments marked dilatation of the right ventricle, suggesting impairment of the circulation, was observed during a period of over-distention of the lungs. Under this condition the systemic blood pressure and, therefore, the pressure in the carotid sinus seemed to have decreased and yet decreased pressure renders the participation of this mechanism in apnea during distention unlikely. Further investigation of this problem is necessary.

If the degree of distention of the lungs exerts important influences on the behavior of the respiratory center, it becomes necessary to keep the volume of the lungs constant in experiments dealing with the regulation of respiration. How sensitive this aspect of the whole mechanism may be is suggested by the fact that functional residual air (Binger) (normal capacity, Rohrer), a factor in establishing the degree of distention, varies with position. It is greater in the upright than in the prone position and this may be sufficient to change the irritability of the respiratory center, and hence the character of respiration.

SUMMARY

1. In dogs, when the chest is laid open, the influence of distention and collapse of the lungs on the duration of the period of apnea has been studied. By taking successive small samples of arterial blood toward the end of the period, the threshold value of the respiratory center was ascertained.
2. Distention of the lungs is responsible for the onset of apnea or prolongs this state if it exists already, and slows the respiratory (thoracic) rhythm.
3. In distention, respiratory movements do not occur until the oxygen concentration in the blood is lower and the carbon dioxide higher than in collapse.
4. Distention of the lungs may be said, therefore, to influence respiration in the sense of decreasing the irritability of the respiratory center.
5. The influence of pulmonary distention disappears after bilateral vagotomy.

PART II. THE RELATION BETWEEN PULMONARY AND THORACIC MOVEMENTS. Traube (14) has pointed out that distention of the lungs is followed on each occasion by an expiratory movement, and collapse of the lungs by an inspiratory one. This observation has been confirmed several times. Breuer also found that this relation exists provided the individual insufflations are large enough to create appreciable differences in the volume of the lungs. If, however, the insufflations are so small and rapid that no significant change in volume occurs, the thoracic movements are entirely independent of the rhythm of insufflation. This complex or phenomenon was generally regarded as evidence of the auto-regulatory mechanism described by Hering and Breuer. In the literature, and in our own experiments, we find that both in distention and in collapse, regular thoracic movements may occur without any change in pulmonary volume, an occurrence which is contrary to the behavior one would expect if the Hering-Breuer theory were wholly correct. This study was begun, therefore, in a search for other regulatory mechanisms.

In the preceding part of this paper it was shown that distention of the lungs decreased the sensitivity of the respiratory center, and changed the respiratory rhythm. We wished, therefore, to investigate the extent of the influence of distention of the lungs on the relation between their movement and the motion of the thorax. Observations were first made on the relation of spontaneous movements of the thorax to movements forced upon the lungs by varied conditions of artificial ventilation. The general operative procedure was similar to that described in part I.

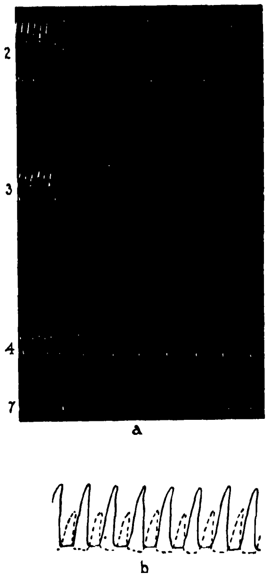


FIG. 7. The time relations are shown during artificial ventilation of a dog. The curves record events similar to those described in figure 1 except that curves 1, 5 and 6 are omitted. In (a) the original curves are reproduced; in (b) a redrawing of curves 1 and 2 in their proper relations.

RESULTS. If the volume of the ventilation is constant, but if the dog is kept *slightly* under-ventilated, the number of movements of the thorax depends on the number of movements of the lungs. Under these circumstances it is easy to demonstrate the relation described by Traube (14, 15) (fig. 7). The motions of the thorax take place in the intervals between insufflations of the lungs. In the experiments if, for example, the ratio of movements of lungs to movements of thorax was, under a certain set of conditions, 1 to 1, this ratio changed when the number of insufflations, having been 16 per minute, was decreased to 11; there were then two thoracic movements instead of one (fig. 8). When the insufflations were stopped (incident to manipulation of the pump) the rate of the thoracic movements increased. If the rate of insufflations was raised to 16 per minute the 1 to 1 rhythm was resumed at once. A reduction of the rate to 9 again occasioned a 2 to 1 rhythm. Following this slow rate of movement of the lungs, there were found during the periods of collapse, equal and regular thoracic movements (fig. 8).

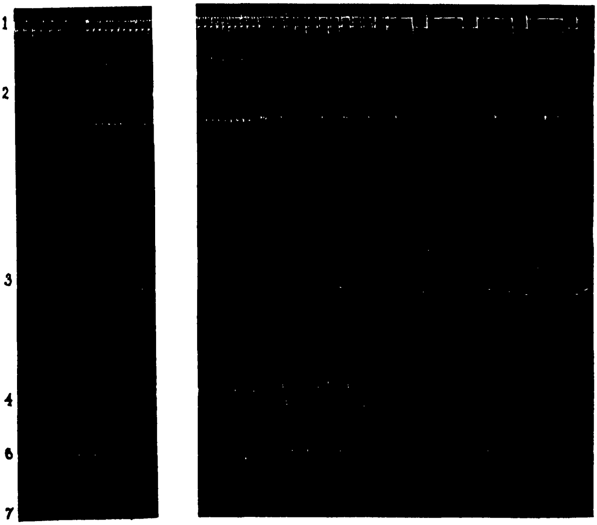


Fig. 8. The effect is shown of changes in frequency of ventilation (strokes of the pump) on the thoracic and abdominal motions of a dog. The division of the curve into two parts indicates an interval of 2½ minutes. At first, reading from left to right, for each stroke of the pump there are two of the thorax. When the strokes of the pump are increased in frequency the ratio of pump to thorax is 1 to 1 preceded and succeeded by short periods when artificial ventilation ceased and a few rapid spontaneous thoracic motions are observed. When the rate of the pump is again reduced, the ratio returns to 1 to 2. Arrest of the pump occasioned once more rapid thoracic motions. The curves record events similar to those described in figure 1 except that the signal magnet for recording the time of maneuvers is omitted.

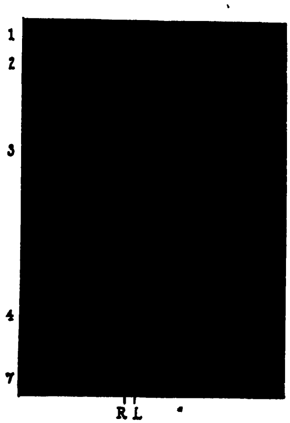
Following section of the vagi these relations are at once destroyed.

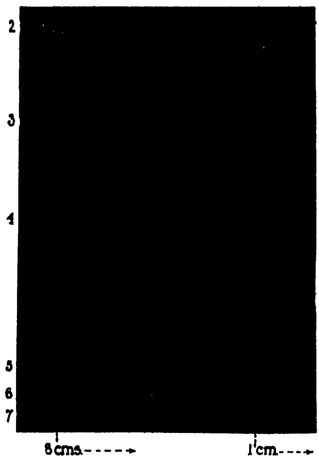
If at first a 2 to 1 rhythm is present, this changes immediately after bilateral vagotomy to an unrelated rhythm of about 9 thoracic movements to 6 distentions of the lungs (fig. 9).

DISCUSSION. Our experiments confirm the observation that regular thoracic movements may occur without changes in the volume of the lungs. They also confirm the fact that during each period of insufflation no thoracic movements need take place. Insufflation acts apparently as an inhibitor of the respiratory center. Attempts to analyze the relations as they are exhibited in our curves, in the light of the Hering-Breuer theory, meet with failure. On examination of the curves one finds that the beginning of distention of the lungs causes an expiratory movement of the thorax, and that an inspiratory motion occurs either at the point of maximum distention or on the arrival of the lungs at the position of collapse. One difficulty is in our curves; artificial expiration is so nearly instantaneous because of the low expiratory resistance, that comparison during this phase is impossible. That the maximum distention should cause an inspiratory movement would be directly opposed to the Hering-Breuer theory, and that return to the position of collapse should likewise stimulate inspiration is unlikely, since the same degree of distention (collapse) would then cause both inspiration and expiration. It seems unnecessary to continue this discussion; it is desirable, however, to emphasize the observation that thoracic movements cease during the active phase of artificial ventilation and take place only during the intervals between insufflations.

So far as the relation of the function of the vagus nerves to these phenomena is concerned, one can conclude no more than that inhibition of thoracic movements during each period of artificial distention of the lungs occurs only when the vagi are intact as vagotomy experiments show.

Since distention of the lungs has been shown to decrease the irritability of the respiratory center, the question arises as to how far, and by what means, the degree of distention controls the relation between movements of the lungs and the thorax. A given concentration of oxygen and carbon dioxide in the blood can either inhibit or give rise to regular thoracic motion depending on whether the lungs are distended or in a state of collapse respectively. Experiments were, therefore, carried out in order to show how, without change in respiratory minute volume, alteration of the volume of the lungs affects the thoracic movements.





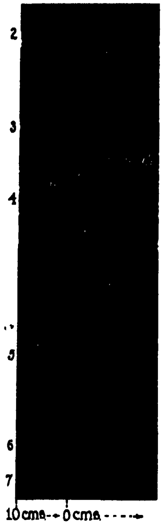


FIG. 9 FIG. 10 FIG. 11

FIG. 9. The effect is shown on pulmonary and thoracic motions of cutting the vagus nerves. The curves record events similar to those in figure 1, except that the signal magnet to record maneuvers and the ten-second signal are omitted.

FIG. 10. The effect is shown 1, of increasing the resistance to expiration to 8.0 cm. water, and 2, of decreasing to 1.0 cm. water. The curves represent events similar to those in figure 1, except that curve 1 representing strokes of the respiration pump is here omitted and that, in the topmost curve in this figure (curve 2) inspiration is a downstroke.

FIG. 11. The effect is shown on pulmonary and thoracic motions of reducing suddenly the resistance to expiration, and so the degree of pulmonary distention, from 10.0 cm. to 0.0 cm. water. The curves represent events similar to those in figure 1 except that the signal for the respiration pump is omitted. In the topmost curve (curve 2) inspiration is an upstroke as in figure 1.

If the animal was *markedly* under-ventilated, increase in the volume of the lungs sometimes resulted in bringing about decrease in extent of thoracic movements, but their frequency and their temporal relation to pulmonary movements remained unchanged. Sometimes the changes in diaphragmatic tone described by Head (5) and Hess (16) could be observed. If, however, the animal was so *slightly* under-ventilated as just to avoid apnea, moderate distention produced marked slowing of respiratory movements while considerable distention (fig. 10)[2] produced apnea.

With fixed respiratory minute volumes, the effect of increasing or decreasing distention of the lungs on the thoracic movements is dependent in part on the magnitude of the minute volume. The observation that slightly under-ventilated animals become apneic with increased distention of the lungs, may be explained as due to consequent diminished irritability of the respiratory center. If there were a doubt concerning the meaning of these experiments, it would arise from the fact that the blood exhibits slightly greater oxygen and slightly lower carbon dioxide concentrations during periods of distention of the lungs than during control periods, the respiratory minute volume remaining the same. Because apnea occurs at once, however, it seems unlikely that change in the blood is the decisive factor in bringing about the period of apnea.

Finally, experiments were designed to show whether thoracic movements depended on the extent of the movements of the lungs. Animals, prepared as usual, were much under-ventilated. By means of markedly increased artificial expiratory resistance, great increase in the volume of the lungs was obtained.[3] The expiratory resistance was then so greatly reduced, the respiratory minute volume being kept constant, that the maximum volume of the lungs in inspiration was smaller than the minimum volume had been during expiration (fig. 11). Since, when the lungs were distended, the expiratory phase failed to inhibit the respiratory center, it seemed likely that when they were smaller in full inspiration than they had been before during expiration, the degree of their distention would fail to influence the respiratory center and so bring about dissociation between motions of the lungs and motions of the thorax. But this result did not occur. The motions of the lungs and thorax remained associated, even as they had been before (fig. 11).

---

[2] This animal, now being described, under the usual conditions of ventilation moved his thorax only during every second or third instead of during every pause between insufflations. Although the numerical relation between distention of the lungs and thoracic movements was somewhat different, the underlying principle of coördination remained undisturbed, since thoracic movements took place only during the intervals between distentions of the lungs.

[3] From the curve recording intra-tracheal pressure, inferences regarding the volume of the lungs can be made directly. Following over-distention, a return to a smaller size would mean that the initial elasticity had been recovered. Under these conditions the volume for identical pressures would be less.

This experiment is valuable because although the efficiency of the same respiratory minute volume would be slightly lowered by decrease in volume of the lungs, resulting in a tendency toward increase in acidity of the blood, a tendency which would be more favorable for dissociation of pulmonary and thoracic movements, dissociation did not occur. Since these movements continued to be associated it appears that the links in the chain of causation—degree of distention of the lungs, degree of irritability of the respiratory center, inhibition of respiratory movements—are interdependent to a moderate degree only. The static factor, the degree of distention of the lungs, therefore, is not the occasion for sending relevant centripetal impulses to the respiratory center. One can conclude, in consequence, that impulses dependent upon *movement* itself of the lungs, are concerned in this association. Which mechanism, precisely, movement of the lungs sets into operation, whether it is stretching of the tissue itself, the influence of the air stream on the bronchial mucous membrane or even periodic changes in pulmonary blood pressure, is unknown. The stronger of these unrecognized influences was called into play in some of our earlier experiments (fig. 1b). After the acidity of the blood had risen during apnea above a point where it was certain to stimulate the respiratory center, it was effective in initiating motion even though the degree of distention of the lungs was great enough, under other circumstances to inhibit thoracic movements altogether. Associated motions began again immediately when the lungs were set in motion artificially (though the degree of their distention was still great). No conclusion more searching can be drawn than that these influences, the influence of acidity of the blood and that of motion of the lungs, are more profound than centripetal impulses arising from the degree of distention. That this inference is just is fortified by experiences with animals in which, though so markedly under-ventilated that distention of the lungs was executed without exhibiting its usual inhibitory influence, complete association persisted nevertheless.

CONCLUSIONS

1. In dogs with widely open pneumothorax, the association of inspiratory and expiratory movements of the thorax and distention and collapse of the lungs results from inhibition of the respiratory movements during each insufflation of the lungs.
2. This association cannot be fully explained by inhibitory impulses arising from the distended lungs, for their state as shown in part I of this paper is able to occasion apnea only under certain conditions.
3. Motion of the lungs or, more properly, one of the factors of motion, stretching of lung tissue, changes in alveolar tension or pulmonary blood pressure, motion of the air in the bronchial tubes, appears necessary for this association.

BIBLIOGRAPHY

1. Breuer, J. 1868. Sitzungsb. d. K. Akad. d. Wissensch. Math.-naturw. Kl. ii, lviii, Wien.
2. Haldane, J. S. 1922. Respiration. Yale Univ. Press. New Haven.
3. Lockenberg, E. 1873. Verhandl. d. Physikal-Med-Gesellsch. in Würzburg. Neue Folge iv, 239.
4. Guttmann, P. 1875. Arch. f. Anat., Physiol. u. wissensch. Med., p. 500.
5. Head, H. 1889. Part I. Journ. Physiol., x, 1.
6. Christiansen, J., and J. S. Haldane. 1914. Journ. Physiol., xlviii, 272.
7. Filehne, W. 1879. Arch. f. exp. Path. u. Pharm., x, 442, xi, 45.
8. Knoll. 1925. Quoted by G. Bayer. Handb. d. norm. u. path. Physiol., ii, 455.
9. Wieland, H. 1916. Arch. f. exp. Path. u. Pharm., lxxix, 95.
10. Van Slyke, D. D., and J. M. Neill. 1924. Journ. Biol. Chem., lxi, 523.
11. Hastings, A. B., and J. Sendroy, Jr. 1924. Journ. Biol. Chem., lxi, 695.
12. Heymans, J. F., et C. Heymans. 1927. Arch. Internat. d. Pharmacody. et de Therap., xxxiii, 273.
13. Heymans, C., J. J. Gouckaert, et L. Dautrebande. 1930. C. R. de la Soc. de biol., cv, 881.
14. Traube, L. 1846. Beit. z. exp. Path. u. Physiol., i, 65.
15. Traube, L. 1846. Beit. z. exp. Path. u. Physiol., ii, 91.
16. Hess, W. R. 1930–31. Pflüger's Arch., ccxxvi, 198.

[Reprinted from THE JOURNAL OF CLINICAL INVESTIGATION, November, 1932, Vol. 11, No. 6, pp. 1321–1341]

STUDIES ON CHEYNE-STOKES RESPIRATION

BY ALBERT J. ANTHONY, ALFRED E. COHN, AND J. MURRAY STEELE

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 6, 1932)

PART I

Changes in Concentration of Gases in the Blood

Cheyne-Stokes respiration is characterized by rhythmic changes in breathing in which periods of respiratory pause alternate with periods of slowly increasing and decreasing respiratory activity. It may be accompanied by phasic variations in the functions of the heart, nervous system, and other organs, and has more recently been found to be associated with periodic variation in the concentration of the gases in the blood. It occurs as a symptom in diseases of the heart and kidneys especially, in meningitis and in encephalitis, and in certain intoxications such as morphine poisoning and oxygen lack. The development of this type of breathing has been ascribed to many causes—disturbances in the gaseous exchange in the lungs, of the circulation, of the activity of the respiratory and vasomotor centers, and to so-called subcortical influences. Objections to all these hypotheses have been entered with the result that no satisfactory understanding of the subject has been attained. It is of value, therefore, to record further observations of Cheyne-Stokes respiration and its attendant or resultant phenomena.

The phenomenon concerning which it seems now important to accumulate more experience, is the behavior of the gases, carbon dioxide and oxygen, in the blood under the circumstances which attend this type of respiration. For this purpose, samples of arterial blood taken from patients exhibiting this abnormality were studied. A few analyses of arterial blood have already been reported by Gollwitzer-Meier (1), Uhlenbruck (2), and Resnik and Lathrop (3). Their studies show that variations in the concentration of the gases in the blood do occur during different phases of respiration[1] and that at some point during the respiratory cycle, oxygen saturation of the blood usually reaches normal limits. The average carbon dioxide content in the blood is reported in some cases to be increased, in others decreased, and in others normal. In former studies only one or two samples of blood were, however, taken; but in order to ascertain the extent of the variation in the concentration of the gases in the blood during a cycle of Cheyne-Stokes respiration it is necessary to collect from a continuous flow of arterial blood, separate, immediately successive samples as rapidly as possible. For this purpose a needle of the type usually used in puncturing arteries was connected with a nine-way stopcock.[2] With its aid, samples could be rapidly obtained. A somewhat similar method has recently been published by Klein (4). The blood was collected under liquid paraffin; the oxygen and carbon dioxide content were estimated by the method described by Van Slyke and Neill (5), and the hydrogen ion concentration according to that of Hastings and Sendroy (6).

Two patients were selected for study in whom Cheyne-Stokes respiration was observed in the hospital over a period of several months, and who, because of decreased mental activity, were not disturbed by the procedure of arterial puncture. A long period of observation was regarded as important in order to be certain of the continuous presence of this type of breathing. As is well known, there are cases in which Cheyne-Stokes breathing occurs in a transient manner, as for example in cases of heart failure and morphine poisoning or in normal individuals during a sojourn in rarefied air. Since the transient type may be distinct from the continuous, it is unknown at present whether the results of the observations now reported are applicable to cases of Cheyne-Stokes respiration in general.

---

[1] In this paper the Cheyne-Stokes *cycle* is described as divisible into a respiratory *phase* and an apneic *phase*.

[2] This stopcock was made of glass and first used in January 1931. Blood entered through an opening in the bottom of the center plug emerging on the side. Eight perforations through the jacket in a circle at the level of the opening in the center plug lead by rubber connections to openings into the lower ends of the glass sample tubes. The sample tubes are held by a stout rubber band against the outer surface of the jacket. The whole apparatus is then filled with paraffin oil. By revolving the jacket with the sample tubes fixed to it, the tubes can be successively and rapidly connected to the arterial needle or cannula.

Case I. The first patient, S. H., male, aged 52, Hospital number 7599, was admitted to the hospital on December 5, 1930. The diagnosis was hypertension and cardiac insufficiency. After a long period, during which he was kept under the influence of digitalis and was obliged to rest in bed, he gradually improved. For about three months before discharge the periods of Cheyne-Stokes respiration had been constantly present; but for the last three weeks, after considerable betterment, they had become intermittent. He then left this hospital.

In the only study made of this patient four samples of blood were taken, the oxygen contents of which varied between 15.1 and 18.5 volumes per cent (79.9 and 98.2 per cent of saturation), while those of the carbon dioxide varied between 42.2 and 50.1 volumes per cent. The number of samples was too small and the time during which they were taken was, in the case of each sample, too long to construct a curve, but the results are shown schematically in Figure 1. They give only an approximate account of what the course of events must have been. The true maxima and minima were undoubtedly greater than the levels actually obtained in the samples, but whatever their magnitude they were probably attained within the period of time during which the samples characterized by the extreme values were collected. The greatest oxygen concentration was found to occur during the first half of apnea. It decreased during the second half of apnea and the first part of the respiratory phase, wherein it reached its minimum and began to increase again. The maximum was reached as before at the beginning of apnea. The concentration of carbon dioxide varied in an inverse direction. The mean concentrations of both gases occurred at about the beginning of the respiratory phase and either mean concentrations or values between this level and the maximal oxygen and minimal carbon dioxide concentrations occurred at the end of the respiratory phase.

Case II. In the second patient, S. G., male, aged 56, Hospital number 7745, admitted April 24, 1931, there had developed first right, then left hemiplegia several years before admission. Pareses of all the extremities, and aphasia still persisted. Arterial hypertension was

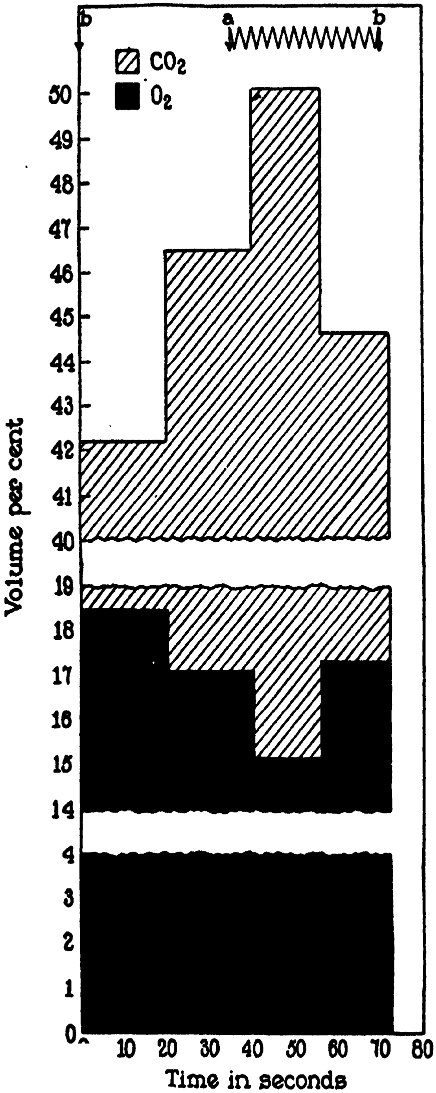


FIG. 1. Chart of the variations in concentration of oxygen and carbon dioxide in blood obtained from the radial artery during a cycle of Cheyne-Stokes respiration (Case I).

Arrows "*a*" mark the onset of dyspnea, arrows "*b*" the onset of apnea.

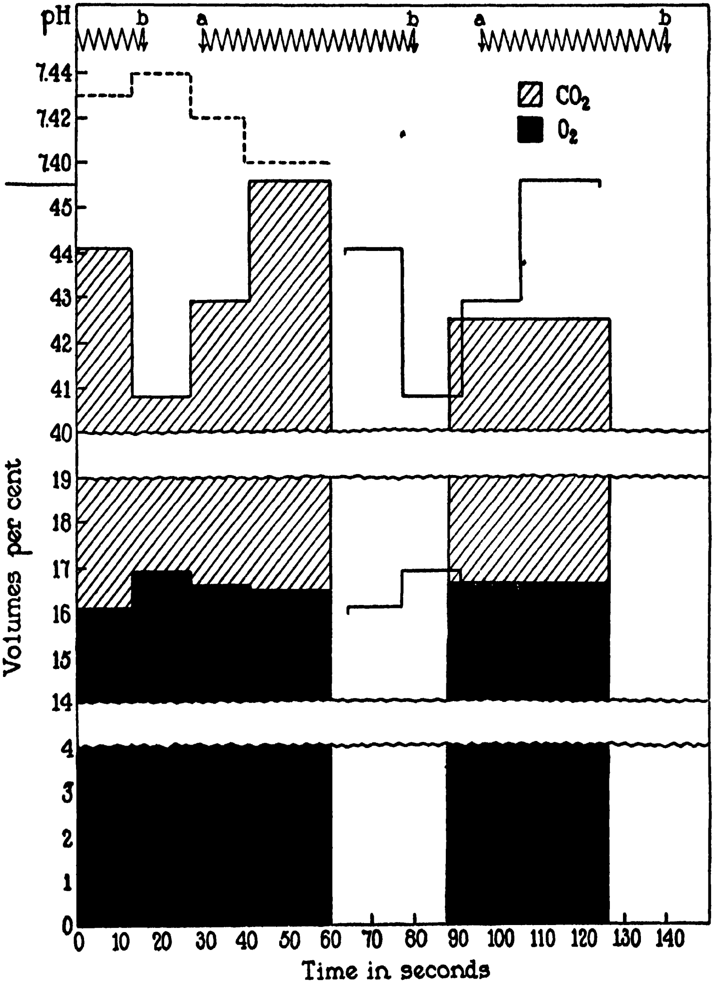


FIG. 2. Chart of the variations in concentration of oxygen and carbon dioxide in blood obtained from the radial artery during a cycle of Cheyne-Stokes respiration (Case II).

A fifth sample of long duration obtained during the next succeeding cycle illustrates the "leveling" effect. The solid line represents the changes in gases of the blood found during the first cycle reproduced in chronological relation to the second cycle. The broken line traces the change in hydrogen ion concentration.

present, but there were no signs of cardiac insufficiency. In the first study (Figure 2) four samples of blood during a single cycle were secured, and one sample during the whole of the next succeeding one.

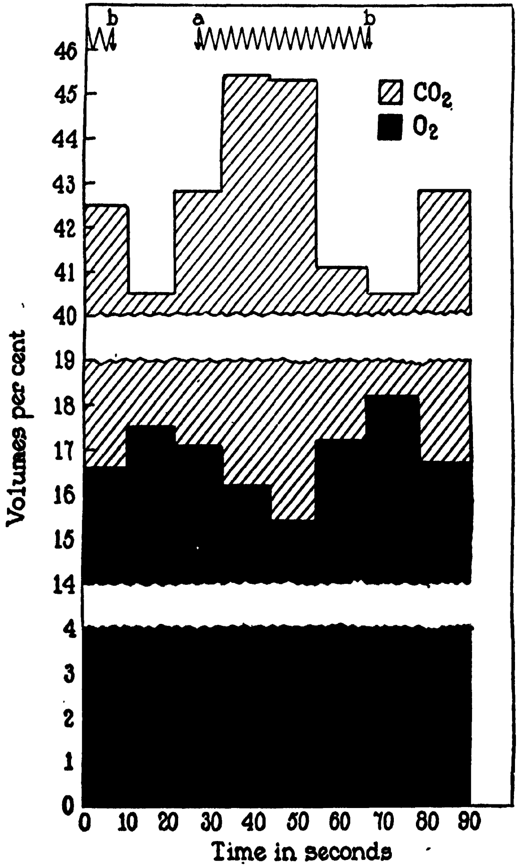


Fig. 3. Chart of the variation of the gases in blood obtained in a later study on the patient shown in Figure 2.

By superimposing the results obtained during the first cycle upon those of the second, it becomes obvious that the longer the time consumed in collecting a single sample, the greater the deviation of the values found from what must have been the actual or extreme values. The oxygen content varied only between 16.1 and 16.9 volumes per cent (89.1 and 93.0 per cent of saturation) and the carbon dioxide between 40.8 and 45.6 volumes per cent. That in this instance the range of variation in the concentration of the gases in the blood was so much less than in Case I may have been due to the fact that the phases of apnea and dyspnea were shorter. The oxygen minimum lay, however, at the same point as in Case I, but the maximum was found to fall, not in the first part of the apneic phase but during the last few respirations. Respiration therefore stopped when maximal oxygen and minimal carbon dioxide concentrations were reached, while it began when they attained mean values. The oxygen content, furthermore, decreased more slowly than it increased. The hydrogen ion concentration of the blood varied with the changes in concentration of the gases, reaching the peak of acidity (pH 7.40) at the time of the carbon dioxide maximum, and decreasing to pH 7.44 at that of the carbon dioxide minimum.

In the second study of this patient (Figure 3), eight samples of blood were taken during two cycles of Cheyne-Stokes breathing. The oxygen content varied between 16.6 and 18.2 volumes per cent (83.7 per cent and 98.7 per cent of saturation), the carbon dioxide between 40.5 and 45.4 volumes per cent. That these changes were greater than those obtained on the previous occasion may have been due to the greater length of the Cheyne-Stokes cycles, or to the shorter time during which each sample was collected. The time at which carbon dioxide was at its maximum and oxygen at its minimum bore the same relation to the respiratory cycle as in the previous observation in this patient. The oxygen maximum lay in the first part of apnea. Concentrations of both gases were approximately near the mid-level when the respiratory phase began. In this study too, oxygen concentration decreased and carbon dioxide concentration increased more slowly than these increased and decreased respectively.

DISCUSSION

In these observations the minimal concentration of oxygen in blood taken from the radial artery occurs constantly during the respiratory phase of the Cheyne-Stokes cycle, and the maximum at the end of this phase, or at the beginning of apnea. The maximal and minimal concentrations of the carbon dioxide occur at the time of the minimal and maximal concentrations respectively of oxygen. Similar results have been reported by Klein (4).

In order to set forth accurately the relations in time between the Cheyne-Stokes cycle and the concentration of gases in the blood leaving the lungs, one must recall the fact that any given concentration at this site precedes its arrival at the radial artery by the time of transmission, which being but two or three seconds, changes the relations only by insignificant amounts. By referring to Figure 3, it becomes apparent that the first few small respirations have little effect on the gases of the blood. The peak of saturation with oxygen is attained, moreover, just before or immediately upon the end of respiration, and indicates that the last few respirations also are quite ineffective.

Because of the different conditions attending the exchange of oxygen and of carbon dioxide between blood and lungs, the changes in their concentrations, though always opposite in direction, are not always inversely proportional. Aside from this relation, the amount of each gas present influences, as is well known, the concentration of the other. The question arises, therefore, how far the concentrations of gases in the blood are determined by variations in alveolar tension, and how far by the varying concentrations of the gases present in the blood itself. This problem has its importance, but the facts which are known are insufficient to analyze the processes which are involved.

The concentration of hydrogen ions in the blood shows changes consistent with the variations found in that of carbon dioxide. These variations are, however, relatively speaking small, due to the presence of the usual buffers of the blood. Since it is known that hemoglobin takes up one-half molecule of carbon dioxide for each molecule of oxygen lost, without change in hydrogen ion concentration, the actual range of variation is still smaller than might be expected because decrease in oxygen takes place simultaneously with increase in carbon dioxide.

If changes in concentration of gases or of hydrogen ions in the blood control phases in the Cheyne-Stokes cycle, the levels of their various concentrations at the beginning of the respiratory phase take on new significance because it is at this point that, considered collectively, as a stimulus, they may be assumed to have reached the threshold value of the respiratory center. Should this suggestion concerning the chain of events be correct, it would afford in man an opportunity to ascertain the threshold value of the respiratory center. The importance of knowing this value lies in the fact that it indicates a definite stage of irritability, readily discerned by a change from inactivity to activity in the organs controlled by the center. If the composition of the blood which is taken from the radial artery is identical with that which arrives at a corresponding time at the respiratory center (due regard being paid, of course, to differences in distance and in the nature of the arterial channels), the threshold of irritability of the respiratory center to carbon dioxide and oxygen lies at about the mean concentration of these gases in the blood, and also at the mean value of the hydrogen ion concentration, between pH 7.40 and 7.44. The last respiration may be a sign, furthermore, that the stimulus derived from the concentration of the gases in the blood has fallen below the threshold value. The impression has been gained in the course of these observations that the threshold changes; that it is lower when the intensity of the stimulus derived from the blood is decreasing than when it is increasing. The problem of the function of the respiratory center with regard to stimuli of increasing and decreasing strength has been so little studied that for the moment further discussion seems unprofitable.

From studies of the analyses of the gases in the blood alone, an explanation of the mechanism of Cheyne-Stokes respiration is not to be expected. For this reason objections may be raised to the conclusions of Klein. His observations and the results he obtained from them are, however, in close accord with those now described. By locating, in the phases of the Cheyne-Stokes cycle the instants of maximal and minimal saturation of oxygen in blood taken from the radial artery, he demonstrated that the oxygen saturation was lowest at about the height of dyspnea and greatest at the beginning of apnea. He then showed, by means of injections of Congo-red into an arm vein that, in patients with Cheyne-Stokes breathing, 18 to 22 seconds passed before the dye appeared in the radial artery, while in normal individuals only 8 to 12 seconds were required. From the increased time of flow he calculated the time at which the blood in the lungs would be most and when least saturated with oxygen. Maximal saturation (in the lungs) occurred at or just before the height of the respiratory phase, but breathing continued until blood saturated with oxygen arrived at the respiratory center. Respiration then stopped. The response of the respiratory center delayed by the slow rate of circulation shown by the experiments with the dye with respect to conditions at the site of ventilation (the lungs) he terms "Nachhinken." After complete saturation of the blood in the respiratory phase, over-ventilation, he believes, occurs with loss of carbon dioxide. Apnea then follows. If the time required for blood to flow from lungs to center is so important a factor as believed by Klein in maintaining periodicity of respiration, changes in the rate of flow should alter the length of the respiratory cycles. But this relation does not exist. If it did, the breathing of normal persons should be periodic in character, the cycles, of course, being shorter since the time required for their blood to reach the respiratory center is but half that found by Klein in Cheyne-Stokes respiration. There are, besides, cases in which the time of flow is prolonged without bringing on Cheyne-Stokes respiration. If a decreased rate of flow were, moreover, responsible for cyclic breathing, the method by which the cycles should disappear is by becoming shorter and shorter. This, however, is not the case in our studies. When increased concentrations of carbon dioxide were administered during Cheyne-Stokes respiration, the cycles remained of about the same length and their disappearance was occasioned by gradual decrease in the intensity of the mid-point of the respiratory phase and by the gradual encroachment of this phase upon the phase of apnea. When, on the other hand, oxygen was administered, the cycles attained twice the usual duration. There is no evidence at hand, however, to show that the circulation time is prolonged by oxygen. Uhlenbruck also speaks of "Nachhinken" of the respiratory center as a result of local difficulties in circulation or in the diffusion of gases.

If, instead of assuming that the time of arrival of blood at the respiratory center and at the radial artery are simultaneous, various intervals of time are assumed and the resulting relations between the beginning of respiration and the concentrations of gases in the blood at the respiratory center are examined, it becomes possible to distinguish on a curve representing changing values of the stimulus of the blood, which portion is capable of arousing the respiratory center. In these observations at the beginning of the respiratory phase the blood in the radial artery was found to have a composition represented on this curve by "*a*" (Figure 4). Should blood leaving the lungs at the same time, and having the same composition, not arrive at the respiratory center until, for example, about 15 seconds later, it would have the same composition as that obtained from the radial artery 15 seconds before the respiratory phase began, that is to say, one similar to "*b*"; or, should the difference in time be greater, a composition similar to "*c*" or "*d*." But these assumptions are all physiologically improbable since blood of low carbon dioxide concentration, having weak stimulating value, would then be associated with respiratory activity, while that of much greater concentration of carbon dioxide, with apnea.

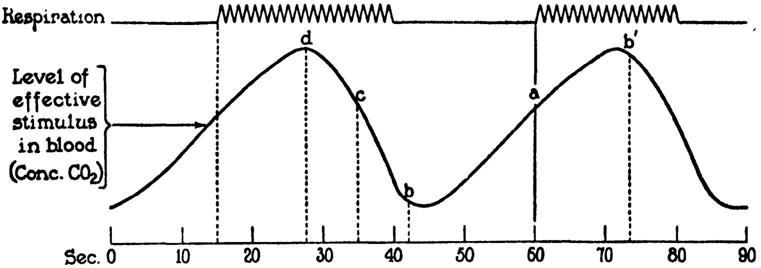


FIG. 4. Schematic representation of various relations of the level of stimulus in the blood (concentration of carbon dioxide), to the phases of the Cheyne-Stokes cycle.

The time relations as drawn are based on the composition of blood obtained from the radial artery. Imagine the respiratory curve shifted to the right so that the beginning of the respiratory phase falls at *b′*, 13 seconds after *a* instead of at *a*, in order to obtain the relation between blood in the capillaries of the lungs to the phases of respiration if the time of blood flow from lungs to radial artery were as great as 13 seconds: or to the left to *b*, *c* or *d*, in order to obtain the relation between blood in the respiratory center and the phases of respiration if blood took 18, 25 or 32 seconds longer to reach the medulla than the radial artery.

On this calculation the arrival of blood of identical composition at the radial artery and at the respiratory center cannot differ by more than 10 or 15 seconds.

Should blood from the capillaries of the lungs take, for example, about 15 seconds to reach the radial artery, it would have at the beginning of respiration the same composition as that obtained from the radial artery 15 seconds after respiration began, that is to say a composition similar to "*b'*" (Figure 4). But this relation is likewise physiologically improbable because the blood in the lungs would then exhibit the effects of ventilation (decrease in content of carbon dioxide) before respiration began. Such a situation is illustrated in Figure 4 where "*b'*" would necessarily represent the blood in the lungs at the beginning of the respiratory phase if it took as long as 15 seconds to reach the radial artery. The interval of time cannot be greater than this in our observations. From the location of these limits it becomes apparent that the values actually found to be associated with the onset of the respiratory phase lie in the mid-zone of the portion of the curve which is capable of arousing the respiratory center.

SUMMARY

By collecting a series of samples in immediate succession and during the briefest periods consistent with securing amounts sufficient for analysis from the continuous stream of blood flowing from the radial artery, it has been possible to analyze the influence of Cheyne-Stokes breathing on the concentration of the gases in the blood.

In the blood drawn from the radial artery, maximal oxygen and minimal carbon dioxide concentrations occur during the end of the dyspneic or first part of the apneic phase. The minimal oxygen and maximal carbon dioxide concentrations occur during the earlier part of the respiratory phase. At the beginning of active respiration, the gas concentrations lie at about a mean value.

PART II

Observations on the Effect of Inhaling Oxygen and Carbon Dioxide

Oxygen lack of the respiratory center is often regarded as the cause of Cheyne-Stokes breathing. This condition is ascribed in some cases to a lack of oxygenation of the blood in the lungs, in others to anoxemia produced by undue slowing of the general or local circulation, and in still others to difficulty in the passage of blood in the respiratory center. Opinions such as these are based on observations of Cheyne-Stokes breathing in normal persons at great elevations above sea level, on

Cheyne-Stokes respiration brought about by the inhalation of gas mixtures low in oxygen, and on clinical investigations of persons exhibiting the Cheyne-Stokes syndrome. The theory is supported, furthermore, by reports of improvement in condition following the administration of oxygen. Several authors (7, 8) have described cases in which Cheyne-Stokes respiration was brought to an end and regular breathing reestablished by the inhalation of oxygen as well as by the inhalation of carbon dioxide. Recently, Uhlenbruck (2) reported that the result with oxygen was transient, regular breathing occurring during the first few moments of administration only. Because the theory of oxygen lack is important both in attempting to analyze the nature, and in the therapy of Cheyne-Stokes respiration, its effect on the form of Cheyne-Stokes breathing was studied in detail.

The administration of oxygen was effected in some instances by inhaling from a mask connected through a rubber bag with an oxygen tank, in others, by enclosing the patient in an oxygen tent. The most satisfactory method was to place the patient in an oxygen chamber. We place special emphasis on the long duration of observation which the last mentioned method permits. Observations made when a mask is used are unsatisfactory because of increase in the dead space and the consequent possibility of rebreathing carbon dioxide, and also because of the danger of leak about the mask. Another objection results because patients sometimes change the type of breathing when a mask is applied to the face. Records of respiration were made by a Marey tambour connected by tubing with two rubber bags held in place against the chest by a canvas belt. The transmission was by air. The lever of the tambour wrote in ink on glazed paper moved by a revolving drum. The tracings were used only in calculating time relations; for volumetric measurements they were useless. To secure volume curves a Roth-Benedict spirometer and mask were used. The objections to this method are the same as those mentioned above. A more serious objection, due to progressive decrease in oxygen content of the gas in the spirometer, was overcome by increasing the total volume of the apparatus to about 20 liters. This large capacity was obtained by joining several large glass bottles in series in the respiratory circuit. Oxygen variations under these conditions were less than 3 per cent.

Before the administration of oxygen, records were made of the patients in room air over a period of several hours. In some patients Cheyne-Stokes breathing persisted continuously during this period; in others, it alternated with periods of regular respiration. Transitions between the two were frequently observed in passing between the waking and the sleeping state (Figures 5*a* and *b*). In studying the effect of inhalation of oxygen only patients who were subject to continuous Cheyne-Stokes breathing were observed, except one in whom this type of breathing was present constantly while he was awake.

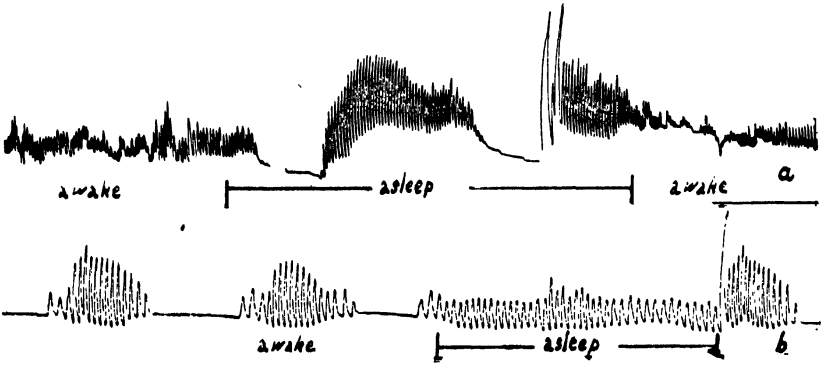


FIG. 5. Kymographic tracings of transition to (*a*) Cheyne-Stokes respiration on falling asleep and (*b*) in another individual to continuous breathing on falling asleep.

In this figure as well as in Figures 6, 7, 8, 9, and 12 the tracings have been reduced to four-fifths of their original size.

Carbon dioxide was first administered to some patients in order to secure curves to be used in comparison with those obtained when oxygen was used. Inhalation of 5 per cent carbon dioxide by mask stopped the occurrence of apneic phases after two minutes; only the frequency of respiration then showed periodic variations. As soon as carbon dioxide was withdrawn, Cheyne-Stokes respiration recurred. No after effects suggesting exhaustion of the respiratory center appeared. In an experiment of long duration, carbon dioxide was administered in a gas-tight chamber in which the gas could be slowly increased and accurately analyzed, and in which the oxygen content was maintained

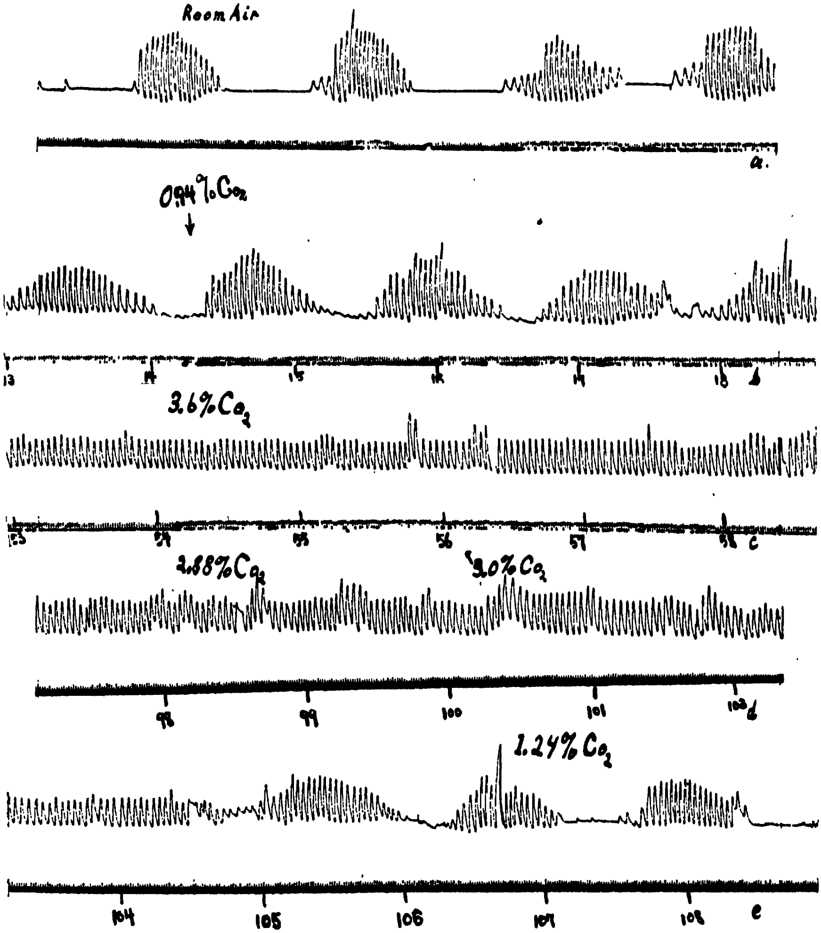


FIG. 6. Tracings of changes in form of respiration showing the gradual development of continuous breathing during the inhalation of increased amounts of carbon dioxide.

Note the gradual encroachment of the dyspneic upon the apneic phase in *b* causing a disappearance of the latter without change in the duration of the whole cycle.

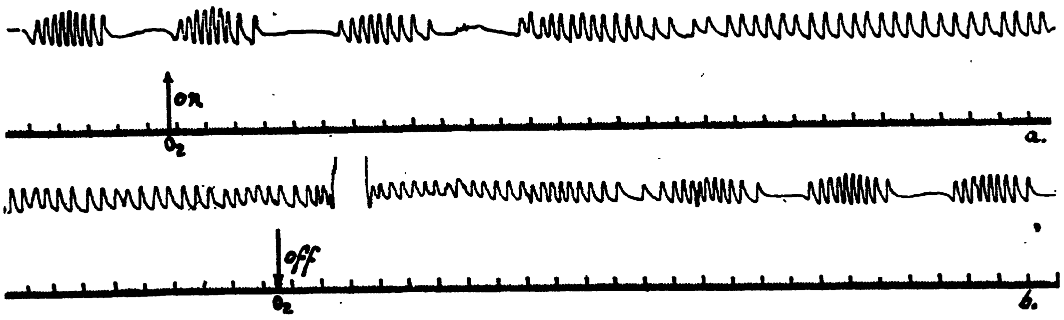


Fig. 7. This tracing shows the only example of continuous breathing observed during inhalation of oxygen which, in this instance, was administered by a mask.

practically constant. By this means the details of the onset of continuous breathing were recorded. A period of control is illustrated in Figure 6*a*. When the carbon dioxide content reached one per cent, the respiratory phases began to increase in duration (Figure 6*b*) while the apneic phases became shorter and began indeed to disappear. The length of an entire cycle did not, however, increase. When the concentration reached approximately 3 per cent (Figure 6*c* and *d*), the breathing was continuous and remained so throughout the whole period of administration lasting one and one-half hours. On discontinuing the administration of carbon dioxide, the transition to Cheyne-Stokes breathing took place. The apneic phases were at first brief, then grew longer, while the respiratory phases grew shorter

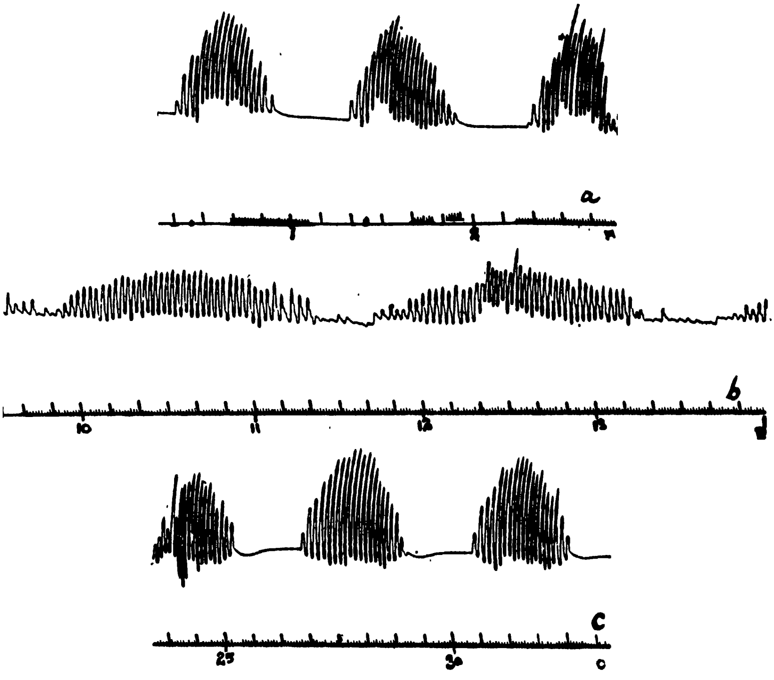


FIG. 8. Tracings *a* and *c* were obtained before and after, and *b* during the administration of 80 per cent oxygen by mask and valve.

The increase in the length of the whole cycle is well shown in "*b*".

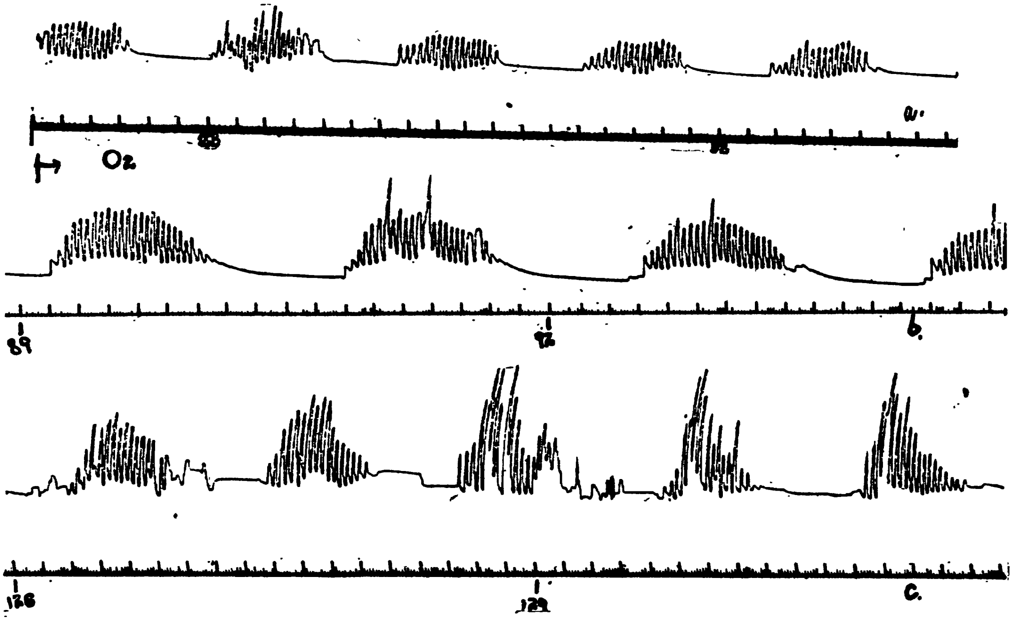


FIG. 9. Tracings *a*, *b* and *c* were taken before, during, and after the administration of oxygen under a tent.

The whole cycle has increased as in Figure 8*b*, but the increase in this instance is mainly due to the greater duration of apnea.

(Figure 6*e*). No after effect suggesting exhaustion of the respiratory center was apparent even after this prolonged period of inhalation.

Once when oxygen was administered for a short time by mask there was a sudden transition to continuous breathing after two minutes (Figure 7). On another day, using the same technique, continuous breathing did not appear but the respiratory phase increased from 37 to 67 seconds, while the duration of apnea did not change appreciably. The whole Cheyne-Stokes cycle, therefore, increased about 50 per cent.

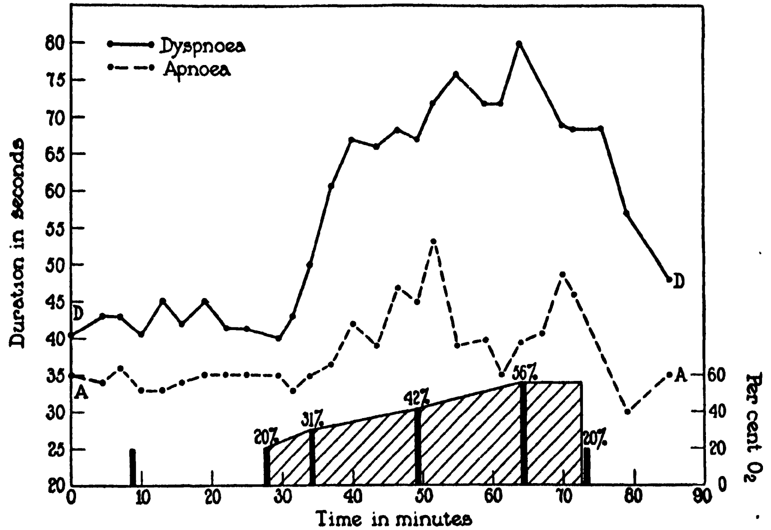


FIG. 10. Chart of the change in duration of apnea and dyspnea during increase in the concentration of oxygen inhaled.

A somewhat similar increase in another instance occurred when a mask fitted with one-way valves was used, and 80 per cent oxygen was inhaled from a large spirometer (Figure 8*a*). In this case a marked increase in the length of the respiratory phase and a decrease in the duration of apnea took place, the whole cycle increasing 75 per cent in length (Figure 8*b*). In the oxygen tent and chamber where the content of oxygen in the air breathed ranged from 40 to 55 per cent, somewhat similar results were obtained except that a decrease in the duration of apnea was never observed. When duration of apnea changed, it always increased in length. An increase in the length of the respiratory phase was always present (Figures 9, 10, and 11).

In one patient curves of the respiratory volume were obtained while the concentration of oxygen of the respired air was changed from 20 to 33 per cent (Figure 12). The respiratory and apneic phases both increased as usual, while the respiratory volume per minute decreased about 10 per cent, an amount just within the limit of error of measurement. The total amount of air respired during one whole cycle increased, however, about 50 per cent. This result was due to increase in length of the respiratory phase as well as to increase in the volume of each individual respiration. By modifying the cycles of his breathing a patient suffering from Cheyne-Stokes respiration may alter, without changing the volume of respired air, the composition of the gases in the blood. It is improbable therefore that measurement of the volume of the respired air would afford reliable information about the oxygen absorbed or the concentration of the gases in the blood.

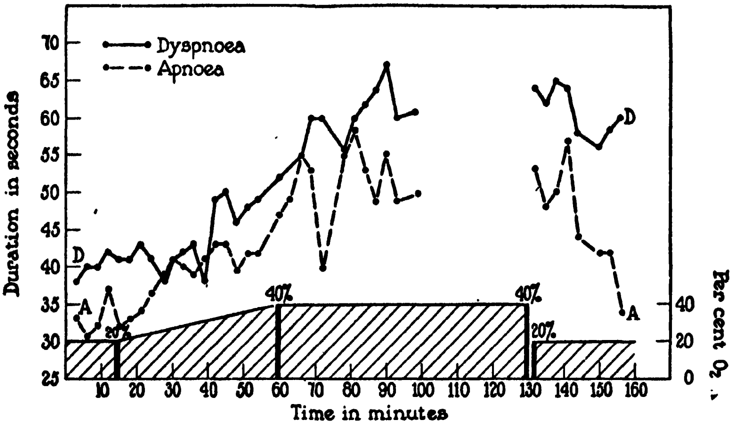


FIG. 11. Chart of the change in duration of apnea and dyspnea during increase in the concentration of oxygen inhaled.

A series of samples of arterial blood were examined during the administration of oxygen for comparison with the results obtained when room air was respired. During its administration the variations in concentration of both oxygen and carbon dioxide in the blood were much less during a respiratory cycle. In none of the samples did the content of oxygen fall below 90 per cent even though the cyclic form of breathing did not cease.

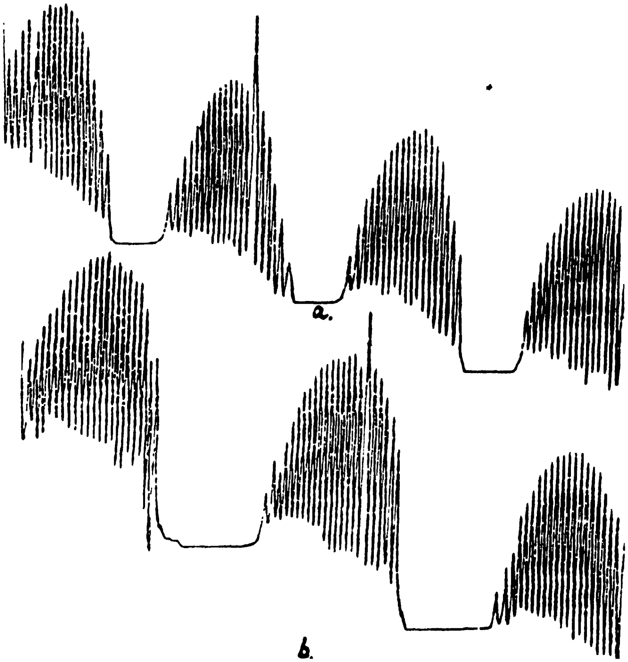


FIG. 12. Volumetric tracing of respiration (Benedict-Roth modified) (*a*) before and (*b*) during inhalation of increased concentration of oxygen.

DISCUSSION

In the present study increase of carbon dioxide content of the atmosphere to which patients, the subjects of Cheyne-Stokes breathing, were exposed, consistently brought about continuous respiration. A similar result was not obtained by the administration of oxygen even if the concentration was increased up to 80 per cent.

With carbon dioxide, the respiratory phase increased and the apneic phase decreased in length, so that the duration of the whole cycle remained unchanged. With oxygen, on the other hand, the length of the respiratory phase increased while that of apnea remained the same or became even longer, so that the duration of the whole cycle was greatly prolonged. That continuous breathing may follow the administration of high percentage of oxygen when given by means of a mask may be due to factors suggested earlier in the paper as objections to this method of administration. It may be due, however, to conditions inherent in the experiment which differ from those obtaining in the use of the oxygen tent or chamber, namely, the occurrence of much greater concentrations of oxygen or the abruptness of the change from atmospheric to higher concentrations of oxygen. Further investigations of the conditions of administration are necessary. These observations do not, we think, permit the inference which has been drawn by others, that inhalation of increased concentrations of oxygen stops Cheyne-Stokes breathing. It is possible, however, to confirm that part of the results of Uhlenbruck's experiences which show that the form of Cheyne-Stokes respiration can be modified by a change in concentration of the gases which are inhaled. It is impossible, therefore, to regard the periodicity of Cheyne-Stokes breathing as an expression of the peculiar rhythm of the respiratory center alone since this periodicity may be modified or even arrested by changing the composition of the inhaled air.

That inhalation of percentages of carbon dioxide higher than normal brings about continuous breathing is probably due to the fact that no matter how great the degree of dyspnea, the concentration of carbon dioxide in the alveolar air prevents the escape of carbon dioxide from the blood in sufficient quantities to allow the stimulating properties of the blood to fall below the threshold value for the respiratory center. Administration of a concentration of carbon dioxide just sufficient to occasion continuous respiration might, therefore, constitute another measure of the irritability of the respiratory center. An analysis of the effect of breathing high percentages of oxygen is more complicated because two factors are involved. In the first place oxygen lack is generally regarded as a respiratory stimulus. When more of this gas is made available, the strength of the stimulus, therefore, decreases; but the stimulating property of the blood may, nevertheless, be increased as the result of oxygenation, since oxyhemoglobin develops stronger acid properties than hemoglobin (9, 10). The two factors act in opposite directions. For instance, by eliminating oxygen lack, one might suppose that the apneic phase would be prolonged, the respiratory phase shortened, and the depth of ventilation decreased. But by reason of the greater acid property of oxyhemoglobin, the effect of better oxygenation might be similar to that of breathing carbon dioxide in that it might result in an increase in the acidity of the blood. Analysis of the nature of the mechanism is also complicated by the fact that every change in the relations of the phases of the Cheyne-Stokes cycle occasions change in the carbon dioxide content of the blood. Further studies of the behavior of the gases in the blood are necessary in the attempt to solve this problem.

If the treatment of Cheyne-Stokes respiration has as its object the restoration to normal of the concentrations of the gases in the blood, then bringing about continuous respiration need not of itself be regarded as improvement, because continuous breathing alone does not necessarily mean that the ventilation is more nearly sufficient. For example, two patients with Cheyne-Stokes respiration were observed in whom the last stage of their disease was accompanied by an increase in cyanosis, and return to continuous breathing at one and the same time. If the object of treatment is to bring about continuous respiration, then inhalation of carbon dioxide in a chamber may be attempted, using the smallest concentration necessary to assure this result. To decrease the rhythmic recurrence of lack of oxygen, the effect of inhalation of oxygen in the chamber may also be utilized as is shown by the analysis of blood of patients while they are in the oxygen chamber.

SUMMARY

The influence on Cheyne-Stokes respiration of breathing varied mixtures of carbon dioxide and air has been studied in periods both of short and of long duration.

Increase in concentration of carbon dioxide in the air inhaled prolongs the respiratory phase, and decreases the apneic phase until continuous breathing appears.

Increase in the concentration of oxygen up to 80 per cent in the air inhaled prolongs the respiratory phase markedly. The duration of apnea is sometimes increased, and sometimes remains constant.

Inhaling oxygen in greater concentrations than that of air does not usually result in continuous breathing.

BIBLIOGRAPHY

1. Gollwitzer-Meier, Kl., Deutsche med. Wchnsch., 1925, li, 980. Ueber das periodische Atmen.
2. Uhlenbruck, P., Ztsch. f. d. ges. exper. Med., 1928, lix, 656. Das Cheyne-Stokessche Atmen.
3. Resnik, W. H., and Lathrop, F. W., Arch. Int. Med., 1925, xxxvi, 229. Changes in the heart rhythm associated with Cheyne-Stokes respiration.
4. Klein, O., Verhandl. d. deutsch. Gesellsch. f. inn. Med., 1930, xlii, 217. Untersuchungen über das Cheyne-Stokesche Atmungsphänomen.
5. Van Slyke, D. D., and Neill, J. M., J. Biol. Chem., 1924, lxi, 523. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement.
6. Hastings, A. B., and Sendroy, J., Jr., J. Biol. Chem., 1924, lxi, 695. Studies of acidosis. XX. The colorimetric determination of blood pH at body temperature without buffer standards.
7. Pembrey, M. S., and Allen, R. W., J. Physiol., 1904–1905, xxxii, (Proc.) p. xviii. Observations upon Cheyne-Stokes' respiration.
8. Douglas, C. G., Haldane, J. S., Henderson, Yandell, and Schneider, E. C., Phil. Trans. Roy. Soc. London, Ser. B, 1913, cciii, 185. Physiological observations made on Pike's Peak Colorado, with special reference to adaptation to low barometric pressures.
9. Hastings, A. B., Van Slyke, D. D., Neill, J. M., Heidelberger, M., and Harington, C. R., J. Biol. Chem., 1924, lx, 89. Studies of gas and electrolyte equilibria in blood. VI. The acid properties of reduced and oxygenated hemoglobin.
10. Van Slyke, D. D., Hastings, A. B., Heidelberger, M., and Neill, J. M., J. Biol. Chem., 1922, liv, 481. Studies of gas and electrolyte equilibria in the blood. III. The alkali-binding and buffer values of oxyhemoglobin and reduced hemoglobin.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, September 1, 1932,
Vol. **56**, No. 3, pp. 345–349]

HISTOLOGICAL STUDIES ON HOG CHOLERA*

II. LESIONS OF THE VASCULAR SYSTEM

BY O. SEIFRIED, V. M. D., AND C. B. CAIN, D. V. M.

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATES 20 TO 22

(Received for publication, April 22, 1932)

As a part of our study of the histological changes associated with the hog cholera virus a description of the findings in the central nervous system has been published (1). In this description attention was called to the lesions in the blood vessels. As the study progressed the importance of these blood vessel lesions became more evident and the present paper is a summary of our findings in this connection.

LITERATURE

Proescher and coworkers (2, 3) found in some organs an acute endangitis combined with a thromboangitis and an end arteritis. Bel (4) described hyperemia of capillaries from which he considers the hemorrhages to be derived but does not refer to special capillary lesions. Roehrer (5) whose work has been done simultaneously with but independently of our own, concludes from the study of the lymph nodes and spleen that the hog cholera virus primarily affects the walls of capillaries and smaller arteries and that other lesions in these organs, such as hemorrhages and necrotic foci, are due to these blood vessel changes. Dide (6) failed to find any characteristic lesions in hog cholera except in the brain. These lesions were similar to those described by Seifried (1) and by Roehrer (5). Eberbeck (7) made no detailed study of the blood vessel system.

Material and Methods

The material for this study is part of that used for the work on the lesions in the central nervous system. Thirty cases of hog cholera were used of which twenty-two were infected by intramuscular injection or by contact and eight were field cases. The experimentally infected animals were killed with chloroform or died 6 to 32 days after inoculation or contact infection. All infected animals had characteristic temperatures and the majority showed more or less typical lesions at autopsy. A few animals, including most of the field cases, did not exhibit lesions that were definite enough to establish a diagnosis. The tissues of a number of cases were cultured immediately after autopsy in order to determine whether secondarily invading bacteria were present.

---

* Presented in part at the meeting of the American Veterinary Medical Association in Kansas City, Missouri, August 25 to 28, 1931; *J. Am. Vet. Med. Assn*, 1932, **33**, 225.

Capillaries and Precapillaries.—In practically all of the organs we studied these vessels usually show the earliest and most pronounced lesions. However, within an individual organ not all capillaries are equally involved. In some organs, for example the kidneys and skin, there is undoubtedly a new formation of capillaries which may be distended with red blood corpuscles or may be empty and collapsed. This is especially true in the lymph nodes. In many cases an enormous swelling and faint staining of the capillary wall is conspicuous even under low power (Fig. 1). With higher magnifications the nuclei of endothelial cells appear markedly enlarged, and because of their lack of chromatin they remain pale or practically unstained (Figs. 2, 4). Frequently the endothelial cells are thrown off or are even entirely lost. Various stains bring out the pale, homogeneous, and hyaline-like appearance of the remaining parts of the blood vessel wall. The thin layers of collagenous or reticular fibers, which closely surround the endothelium, are only partly visible and are undergoing the same changes. The condition is evidently necrotic. It may be so advanced as to change the blood vessel into a thickened hyaline tube, in which cellular or fibrillar elements are no longer visible, or it may lead to a partial or complete occlusion of the capillary lumen (Fig. 3). Less frequently there is a circumscribed proliferation and tumor-like multiplication of endothelial cells, which also show retrogressive changes. In addition to these lesions, the capillary endothelial cells sometimes show nuclear fragments of various sizes and forms scattered around in the more or less thickened or occluded eosinophilic blood vessel wall (Fig. 3). This necrotic process may be restricted to the capillary wall or may extend into the surrounding tissue. Fat in fine droplets can be demonstrated in both capillary walls and perivascular necrotic areas. Thrombosis rarely occurs in the small blood vessels.

Small and Medium Sized Arteries.—Although fundamentally the lesions of the medium sized arteries do not differ from those of the capillaries, they are more striking in appearance and occur less regularly (Fig. 4). Swelling and proliferation of endothelial cells are common (Fig. 5), and the cells show the same retrogressive changes as do those of the capillaries, either appearing as cell shadows or showing various forms of karyorrhexis (Figs. 6, 7). Both progressive and retrogressive changes may be present simultaneously in an individual vessel. The lesions in the intima are almost regularly accompanied by changes, usually less pronounced, in the other structures of the blood vessel wall (Fig. 7). The findings indicate that the endothelial layer is primarily injured by the virus circulating in the blood stream. The lesions of the media and adventitia consist of a separation and distension of the various elements. This is clearly shown by the elastic tissue stain and various silver methods. The blood vessel wall as a whole is markedly thickened by an edematous condition and a multiplication of cellular elements, particularly in the adventitia. Frequently the artery wall is hyalinized and swollen to such an extent that the lumen is occluded (Fig. 4). In many cases the necrotic condition is indicated by fragmentation of nuclei and a pronounced eosinophilia of the other structures, including elastic tissue fibers and other fibrillar elements. Thrombi in such vessels undergo the same process and frequently the necrosis extends into the perivascular tissue.

Larger arteries are less frequently involved, and the changes in them are mainly restricted to the intima. Occasionally veins also may be affected (Fig. 7), and, even more rarely, lymph vessels.

The vessel lesions just described are found in practically all of the cases studied. Their occurrence, character, and degree of severity vary, however, in different organs. In our material they were most pronounced in the lymph nodes, spleen, and kidneys, while in the central nervous system, liver, intestinal tract, skin, and other organs they were less noticeable. It is sometimes difficult to correlate the gross with the microscopical findings.

DISCUSSION

Our study of the vascular system in the various organs reveals the fact that the hog cholera virus, after entering the blood stream, leads to more or less pronounced lesions of the endothelial cells of the capillaries and other blood vessels and that it also affects the outer layers of the smaller blood vessels and capillaries. It should be noted that the type of these lesions is not directly dependent upon the duration of the disease. Furthermore, there are no fundamental differences between the lesions in pure hog cholera cases and those in cases associated with secondarily invading bacteria. We believe that the magnitude of the vascular lesions depends on the virulence of the virus, and somewhat on the presence of secondarily invading bacteria. In acute cases degeneration of the blood vessel walls is prevalent, while in prolonged cases the proliferative character of the endothelial lesions is more evident. Study of our material has shown that secondarily invading bacteria, such as *B. suipestifer*, *B. pyocyaneus*, and bipolar bacteria, aggravate the necrosis of the blood vessel walls and the resulting lesions. We are convinced that the blood vessel lesions constitute a principal feature in the histopathology of hog cholera and offer a satisfactory explanation for the hemorrhages and foci of necrosis that are characteristic of this disease. The changes resulting from the vascular lesions will be described in a succeeding paper.

The vascular lesions in hog cholera, from a comparative point of view, bear a close resemblance to those in pulmonary blood vessels in human influenza, as described by Oberndorfer (8), Le Count (9), and others.

SUMMARY AND CONCLUSIONS

In cases of acute hog cholera the earliest and most pronounced lesions occur in the capillaries and smaller arteries. Larger arteries and veins are less frequently involved. The lesions consist of swelling and proliferation of endothelial cells together with retrogressive changes in them and in the other parts of the blood vessel walls.

The character and degree of these lesions seem to be dependent upon the virulence of the virus and, to some extent, upon the presence of secondarily invading bacteria. We believe that these lesions should be considered a principal feature of the histopathology of hog cholera.

REFERENCES

1. Seifried, O., *J. Exp. Med.*, 1931, **53**, 277; *Ergebn. allg. Path. u. path. Anat.*, 1931, **24**, 554; *Arch. Tierheilk.*, 1932, **64**, 432.
2. Proescher, F. R., and Seil, H., *J. Am. Vet. Med. Assn.*, 1917, **4**, 609.

3. Proescher, F. R., and Hoffman, H. A., *J. Comp. Path. and Therap.*, 1924, **37**,73.
4. Bel, M. P. A., *Rec. méd vét.*, 1927, **103**, 168.
5. Roehrer, H., *Arch. Tierheilk.*, 1930, **62**, 345, 439; 1931, **64**, 125.
6. Dide, M., *Rev. Vet.*, 1930, **82**, 241.
7. Eberbeck, E., *Arch. Tierheilk.*, 1931, **63**, 371.
8. Oberndorfer, S., *Münch. med. Woch.*, 1918, **65**, 810.
9. Le Count, E. R., *J. Am. Med. Assn.*, 1919, **72**, 1519.

EXPLANATION OF PLATES

PLATE 20

FIG. 1. Swelling of capillary walls in a lymph node with faintly stained, degenerating endothelial cells. Hematoxylin-eosin. ×315.

FIG. 2. Partial occlusion of small arteries in the kidney by swelling of the endothelial cells and other structures of their walls. ×400.

FIG. 3, *a*, *b*, *c*. Cross-section through small blood vessel walls showing various stages of thickening, hyalinization, and nuclear fragmentation (semidiagram).

PLATE 21

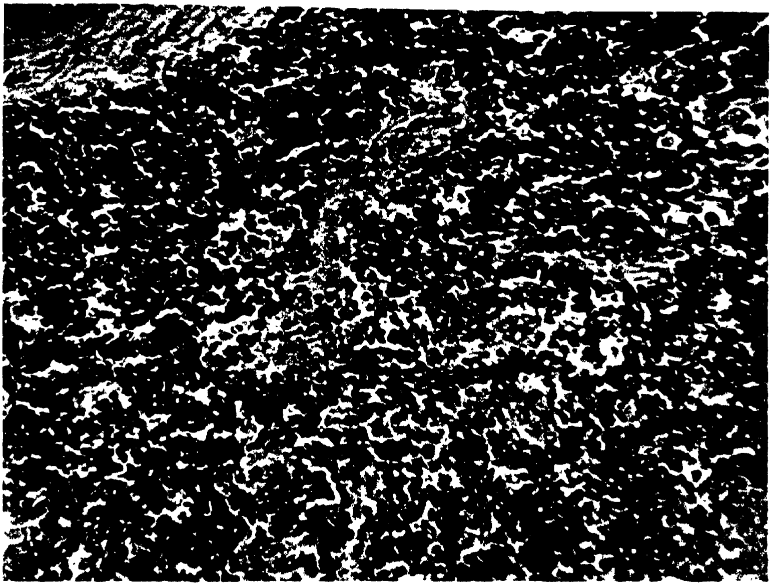
FIG. 4. Swelling, necrosis, and almost complete occlusion of small arteries in the spleen with coagulation necrosis in the surrounding tissue. ×295.

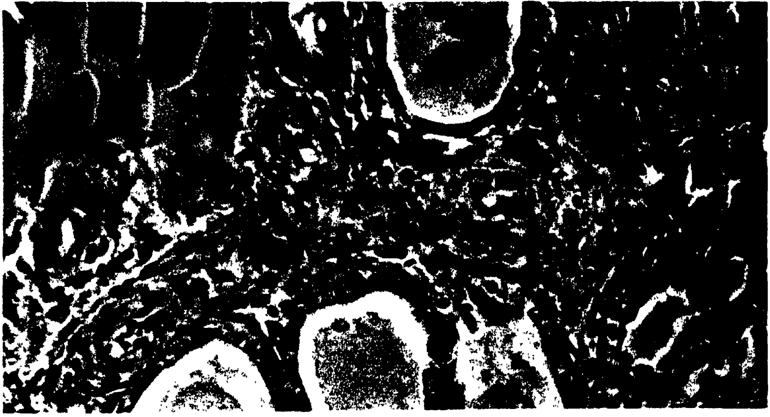
FIG. 5. Multiplication of endothelial cells in a medium sized artery of a lymph node with retrogressive changes in all other structures of the thickened blood vessel wall. Hematoxylin-eosin. ×300.

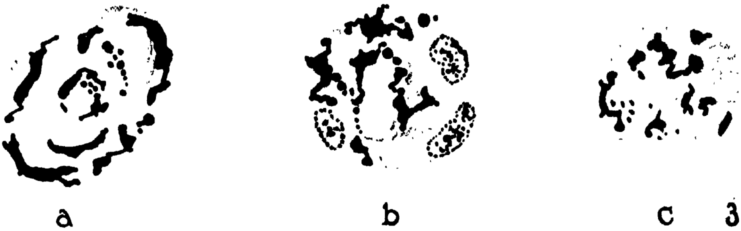
PLATE 22

FIG. 6. Destruction of the endothelial cells of a vein and its branch in a lymph node with a marked perivascular necrosis (karyorrhexis figures of various sizes and shapes). Hematoxylin-eosin. ×300.

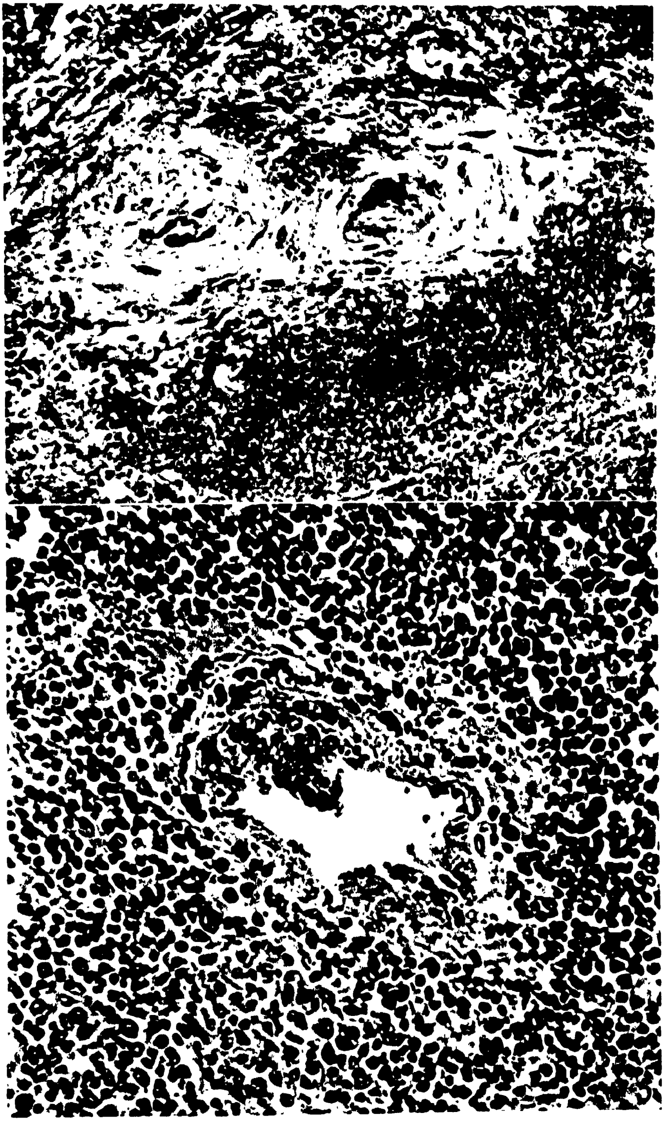
FIG. 7. Partial destruction of endothelial cells in a vein of the liver. Necrotic process extending into the surrounding tissue. ×300.



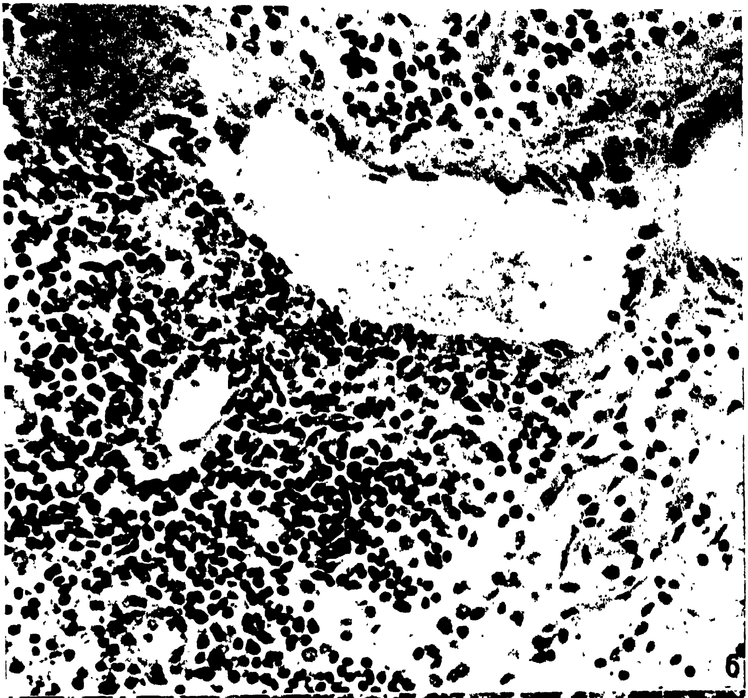




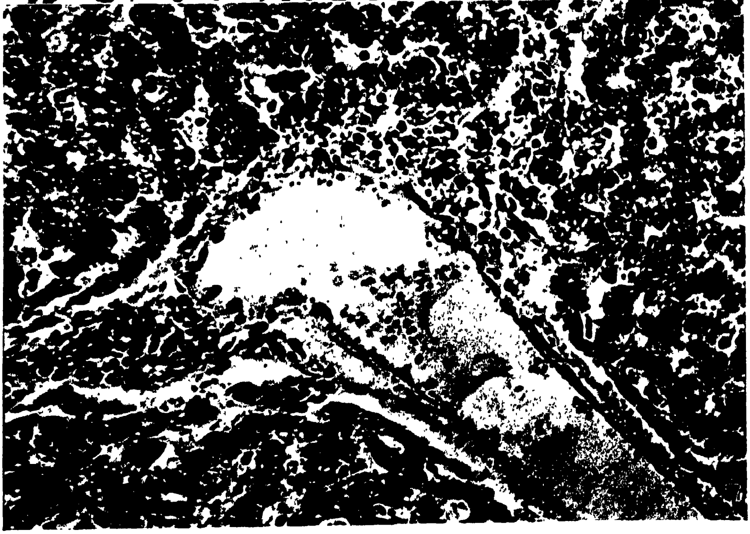
(Seifried and Cain: Histological studies on hog cholera. II)



(Seifried and Cain. Histological studies on hog cholera. II)



6



(Seifried and Cain: Histological studies on hog cholera. II)

[Reprinted from The Journal of Experimental Medicine, September 1, 1932,
Vol. 56, No. 3, pp. 351–362]

HISTOLOGICAL STUDIES ON HOG CHOLERA

III. Lesions in the Various Organs

By O. SEIFRIED, V.M.D., and C. B. CAIN, D.V.M.

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

Plates 23 to 25

(Received for publication, April 22, 1932)

In the preceding paper we have described the changes in the blood vessels of swine infected with hog cholera virus and have stated that these lesions are the principal feature in the histopathology of this disease. In the present paper we propose to discuss in detail the histological changes in the lymph nodes, kidney, spleen, and liver.

A. Blood-Forming Organs

1. Lymph Nodes

The gross lesions in these organs are well known. Three types can be distinguished, (1) swelling and hyperemia, (2) hemorrhagic infiltration especially marked at the periphery, and (3) dense infiltration with red blood corpuscles, indicated by the dark red color of the lymph node parenchyma.

These three types of lesions are usually present in varying degrees in individual cases. In some cases the first type predominates, in others the second and third are more pronounced. Certain groups of lymph nodes are more severely affected than others. In our material there was a general lymphadenitis with the earliest and most characteristic changes in the cervical and thoracic lymph nodes. Although in general the edema and swelling are more frequent in early stages and the foci of necrosis and dark purple color of the lymph nodes usually appear in later ones, there is no rule in this respect. In very acute cases even the reverse may be true.

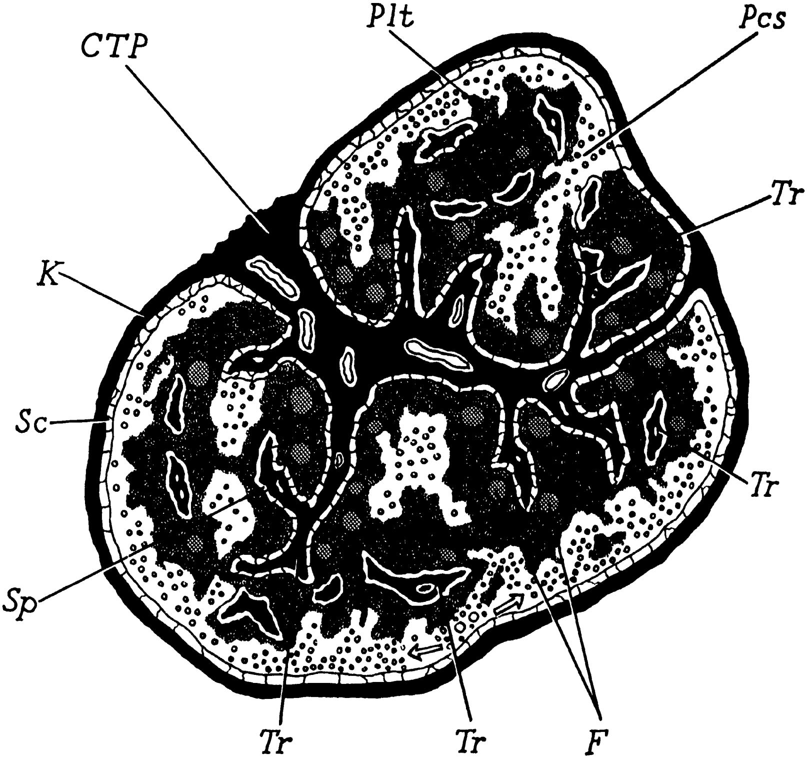
Papers published during the progress of our work indicated that there are various opinions with regard to the fundamental histological findings of the lymph nodes in hog cholera. In peracute cases of hog cholera Bel (1) saw a lymphatic hyperplasia and hypertrophy of the follicles and hyperemia of capillaries from which he considered the hemorrhages in the parenchyma to be derived. In acute and subacute cases there was a pronounced hyperplasia in the lymph nodes which he thought resulted from a proliferation of small lymphocytes and macrophages. Nieberle (2) pointed out that, contrary to the common belief, acute cases of hog cholera are not associated with a hemorrhagic lymphadenitis. He holds the opinion that the red blood corpuscles, found in the lymph nodes, originate in other parts of the body and are carried by the lymph stream into the lymph nodes. Roehrer (3), on the contrary, concludes from the study of his material that the hemorrhages and necrotic foci in the various parts of the lymph nodes are due to lesions in the blood vessels.

Normal Histology of Swine Lymph Nodes.—For an understanding of the lymph nodes in hog cholera it is necessary to consider the normal histological structure. Details will be found in papers by Richter (4) and Trautmann (5); the principal points are brought out in the diagrammatic sketch (Text-fig. 1). The arrangement of the swine lymph node is almost the reverse of that found in other mammals. The lymphoid tissue with its follicles and germinal centers is situated centrally, while the cell-poor substance, which corresponds to the medulla in the lymph nodes of other mammals, is located peripherally but extends in the form of cords into the lymphoid tissue. This cell-poor substance consists of a syncytial network containing numerous capillaries. The connective tissue framework is represented by trabeculae that are rarely connected with the capsule. In the trabeculae are found blood and lymph vessels. There are two sinus systems, one around the trabeculae and the other under the capsule.

The afferent lymph vessels, according to Trautmann (5), enter the node at the connective tissue plate, run through the trabeculae, and open into the peritrabecular sinuses. The lymph only reaches the cortical sinus after passing through the cell-poor substance, except in those rare instances in which the trabeculae come into contact with the cortex.

Lesions in the Lymph Node, Type 1.—There are only slight lesions in the parenchyma. They consist mainly of a separation of cellular and fibrillar elements caused partly by edema, partly, as mitotic figures indicate, by a multiplication of reticulum cells. The edema is most pronounced in the cell-poor substance, especially that immediately surrounding the blood vessels. In a few cases fibrin can be demonstrated in small amounts within the edematous areas. Other changes, occurring irregularly, are: slight perivascular infiltrations in the cell-poor substance, perivascular necrosis of various degrees, swelling and degeneration of reticulum cells, increase of eosinophilic leucocytes, local hyperplasia of lymphoid tissue. Follicles and germinal centers are usually enlarged in size but decreased in number. Peritrabecular and cortical sinuses are entirely free from lesions, except for a slight endothelial desquamation, which may be normal. Trabeculae and capsule are edematous and in some areas show perivascular infiltration with lymphocytes and histiocytes. Perivascular hemorrhages and marked foci of necrosis are not seen in this stage.

Lesions in the Lymph Node, Type 2.—The most outstanding characteristic is the presence of hemorrhages in the cell-poor substance which usually lies in contact with the cortical and peritrabecular sinuses and is also found in cords within the lymphoid tissue (Text-fig. 1). In the latter tissue hemorrhages are not so pronounced. This peculiar distribution of the hemorrhages in the parenchyma



TEXT-FIG. 1. Diagram of a lymph node of swine. *Plt*, lymphoid tissue of the parenchyma; *Pcs*, cell-poor substance of the parenchyma; *Tr*, trabeculae; *F*, follicles; *Sp*, peritrabecular and *Sc*, cortical sinuses; *K*, capsule; *CTP*, connective tissue plate. The distribution of hemorrhages in the lymph node parenchyma in an early case of hog cholera is indicated by the small circles.

explains the marbled gross appearance of such nodes. In fresh hemorrhages the erythrocytes are well stained, while in older ones they show degenerative changes and appear as cell shadows. In larger hemorrhagic areas with dense accumulations of erythrocytes the structures are often indistinguishable. The frequent perivascular arrangement of smaller hemorrhages around changed capillaries and smaller arteries indicates that erythrocytes must escape from these blood vessels. In some instances, however, these vessels show only swelling and proliferation of endothelial cells. Hemorrhages are seldom seen around larger blood vessels even when pronounced lesions are present in their walls. It is remarkable that red blood corpuscles are not found in the peritrabecular and cortical sinuses in these stages even when present in the cell-poor substance in great numbers. In only a few cases do they contain a limited number of erythrocytes either free or phagocytized in sinus endothelium or reticulum cells. In addition to these hemorrhages, more or less marked necrotic foci originating from changed blood vessels may be present in an individual lymph node. These foci may be small, but sometimes, by fusing together, they become so large as to occupy considerable areas of the parenchyma and may be so extensive as to be seen with the naked eye. The distribution of the necrotic areas resembles closely that of the hemorrhages. Only occasionally does the necrotic process affect the sinus walls; as a rule a narrow zone of normal tissue separates necrotic areas and sinuses (Fig. 1). Progressive necrosis involving trabeculae, capsule, and sinuses has been observed in only one case in which secondarily invading bacteria were present. No reactive segregation of necrotic tissue has been seen.

The changes of the remaining structures of the parenchyma correspond to those described in the lesions of Type 1. In the hemorrhagic areas there is a marked hemosiderosis and fatty degeneration of the reticulum cells, but not a striking increase in their number. In necrotic areas or in their immediate surroundings these cells likewise show fatty degeneration or are filled with phagocytized nuclear fragments. Focal hyperplasia of the lymphoid tissue is a rare finding; hyperplasia is found in the majority of cases. Follicles and germinal centers may be reduced in size and number or may be entirely lacking. The changes in the trabeculae and the capsule consist of edema, perivascular cell infiltration, slight hemorrhages, and small foci of necrosis associated with blood vessel lesions.

Lesions in the Lymph Node, Type 3.—Histologically these nodes show an advanced or final stage of the hemorrhagic infiltration found in the lesions of Type 2. Red blood corpuscles may be present in such numbers as to occupy almost the entire cell-poor substance as well as part of the lymphoid tissue. It is remarkable that even in such an advanced stage the peritrabecular and cortical sinuses may sometimes remain entirely free from red blood corpuscles. As a rule, however, the sinuses contain erythrocytes, often in such numbers as to distend the sinuses and rupture the reticular fibers. The presence of red blood corpuscles may cause atrophy of the lymphoid tissue to such an extent that only small islands remain.

These histological findings in the lymph nodes in hog cholera do not support Nieberle's (2) theory of resorption as a complete explanation for the lesions found in these structures.

We feel that the blood vessel lesions (see Paper II of this series) especially those found in the cell-poor substance in the lymph nodes, must be considered the source of edema, hemorrhages, and progressive necrosis in various parts of the lymph nodes.

These findings are in agreement with those recently described by Roehrer (3). An explanation is required for the fact that the hemorrhages are more pronounced in the cell-poor substance than in other parts of the parenchyma. The discovery of Trautmann (5) that the cell-poor substance is a special channel for the passage of the lymph between the peritrabecular and cortical sinuses offers a satisfactory explanation. As soon as they have left the changed capillaries in the cell-poor substance the erythrocytes are carried along with the lymph stream and are distributed in the manner described (Text-fig. 1). When present in great numbers they may enter the sinuses and lymphoid tissue.

2. *Spleen*

Out of thirty autopsies, the spleen showed no lesions in eleven cases and in seven it exhibited hyperplasia which was not different from hyperplasia in other infectious diseases. In the remaining twelve cases anemic infarcts were present.

Recently David and Schwarz (6) emphasized the diagnostic significance of these infarcts. Shortly before our investigations were concluded, Roehrer (7) described histological details of these lesions.

Macroscopically these infarcts appear as dark red, sharply outlined, irregularly shaped foci distinctly protruding above the cut surface of the organ. Sometimes they are a quarter of an inch or more in diameter and they are usually located in clusters on the margin of the spleen. On cross-section they are sharply demarcated from the normal tissue and appear in various forms, the most usual being a wedge shape, the apex of which is orientated toward the hilus (Fig. 2). In addition to these marginal foci there are, in a number of cases, smaller areas of various sizes in the parenchyma similar to the marginal infarcts and likewise surrounded by a hemorrhagic zone.

The histological examination of a great number of these infarcts indicates that they are based on the typical blood vessel lesions, described in Paper II of this series. The most pronounced lesions are in the follicular arteries, especially at the apex of the wedge-shaped infarcts (Figs. 2, 3). Frequently the swelling and hyalinization of blood vessel walls is so enormous as to lead to more or less complete occlusion of their lumen. In some cases thrombotic material is also attached to the endothelium of such blood vessels. In other parts of the infarcts the blood vessels show exactly the same type of lesions. The parenchyma in their immediate neighborhood is found in various degrees of coagulation necrosis (Figs. 2, 3). In the early stages this necrotic process may be restricted to a single follicle but as a rule it extends to the entire infarct including pulpa, follicles, and trabeculae. Fibrin is missing. Hemosiderin is found in the changed blood vessel walls. Towards the hemorrhagic zone of the infarcts the necrosis of the parenchyma is less pronounced. In this area the reticulum contains numerous red blood corpuscles which form an intermediate zone between necrotic tissue and the peripheral hemorrhagic zone. However, this intermediate zone is sometimes entirely missing and in this case the necrotic tissue is immediately surrounded by a dense zone of erythrocytes which is most pronounced immediately beneath the capsule and which is often undergoing retrogressive changes. This explains the pronounced protrusion of the capsule in these areas as well as the destruction of the subcapsular parenchyma and sinuses.

The capsule itself shows in the early stages a slight thickening with a few hemorrhages while in later stages it may, in the infarct areas, become entirely necrotic.

The same infarcts are found in the parenchyma. Often only small microscopic necrotic foci are found. These extend from necrotic blood vessels, especially follicle arteries. In the parenchyma which is free from infarcts, and in spleens which show no gross lesions, slight blood vessel lesions of the type previously described can be demonstrated. They may be associated with perivascular hemorrhages or with slight perivascular necrosis. The most pronounced changes are: a more or less striking increase of pulpa cells, perifollicular hyperemia and hemorrhages of various degrees, infiltration of the Schweigger-Seidel's capillary tubes and trabeculae with red blood corpuscles, and striking aplasia of the spleen follicles.

To sum up it must be emphasized that the infarcts in the spleen in acute cases of hog cholera are caused by primary lesions of the follicular arteries leading to a more or less complete blocking of the circulation in the corresponding areas.

B. Urinary Organs

Although the gross anatomical appearance of the urinary organs, particularly the kidneys, in typical cases of hog cholera is well known, detailed histological information concerning the lesions in these organs is lacking.

According to Proescher and coworkers (8, 9), the lesions in the kidneys correspond to an acute hemorrhagic glomerular nephritis, combined with a tubular nephritis. In peracute hog cholera cases, Bel (1) has described lesions of congestive nephritis with interstitial hemorrhages, changes in the glomeruli and convoluted tubules, proliferation of the endothelial cells and infiltration with mononuclear phagocytes, and scarcity of polymorphonuclear leucocytes. In acute and subacute cases he observed inflammatory reactions in the glomeruli, degeneration of the uriniferous tubules, perivascular infiltration, degenerative processes in the epithelial cells, and cuffs of mononuclear cells around the vessels. Lüttschwager (10) found microscopical hemorrhages in cases that did not show typical gross lesions in the kidneys.

Macroscopically, petechial and ecchymotic hemorrhages are found quite regularly under the capsule and rarely in the medulla and pelvis. However, in some cases there are so few hemorrhages that diagnosis from macroscopic examination alone is difficult if not impossible.

The ureter, bladder, and urethra often show subserous hemorrhages or hemorrhages on their mucous surfaces, but the gross lesions in these organs are far less common than in the kidneys.

Interstitial Tissue.—The small hemorrhages scattered through the cortex and through other parts of the kidneys in practically all cases are regularly associated with the changes in the walls of the affected capillaries. However, the extent of the hemorrhages does not always correspond to the extent of blood vessel injuries. The fact that they frequently occur near the point of entrance of small blood vessels into renal glomeruli (Fig. 4) and that they are often limited to such a distribution in early cases where other changes are not recognizable, suggests that primary injuries occur in the walls of small vessels at these points. Small subcapsular accumulations of blood have a tendency to spread laterally under the capsule so that in cases accompanied by severe hemorrhages a number of these combine to form large subcapsular effusions of blood. Numerous small hemorrhages occurring in the cortex infiltrate interstitial tissue masses of scarcely greater dimensions than glomeruli. Sometimes they are extensive and greatly diffused. Dense accumulations of red blood corpuscles frequently cause the tubules to be widely separated (Fig. 4) and in some areas to be compressed or even collapsed. In more extreme cases the normal structures of the kidneys are obscured by the predominance of erythrocytes. Edema is imperceptible in most cases though it is occasionally present. When present it is more pronounced around affected blood vessels, mainly in the medulla and adjacent cortex.

Not only edema and hemorrhages but also perivascular infiltrations with macrophages (histiocytes) and lymphocytes are seen in the more advanced stages of the disease (Fig. 5). The infiltrating cells, frequently showing degenerative changes, sometimes become so numerous and widespread as to obscure other structures. Necrosis of the interstitial tissues is often continuous with that of blood vessel walls, but necrosis as a result of infarct formation is not found. Proliferation of interstitial connective tissues, occasionally seen, appears to be an extension of the process taking place in the adventitia of the blood vessels. In general the capsule of the kidney shows no significant changes.

Renal Glomeruli.—Capillaries in the renal glomeruli often show the same degenerative changes in their walls as do capillaries in other parts of the kidneys. Hemorrhages in the renal glomeruli occur frequently even in the early stages of the disease process (Fig. 4). Proliferation of the glomerular epithelium is seldom observed. In two cases the renal glomeruli and other structures showed amyloid degeneration. Bowman's capsules exhibit hemorrhages much less frequently than one would expect. Sometimes degenerative changes in the renal glomeruli extend to and include the walls of the corresponding Bowman's capsules. On the other hand, retrogressive changes of the cells lining Bowman's capsules are observed where there are no appreciable changes in the corresponding renal glomeruli.

Tubules.—The tubular epithelium shows varying retrogressive changes in a great many cases (Figs. 4, 5). In a few instances the tubules contain red blood corpuscles and fragments of corpuscles. These may have been caused by hemorrhages in the renal glomeruli or, in advanced stages of tubular degeneration, by the extension of interstitial hemorrhages into the tubules (Fig. 5). In some areas the degenerative lesions of the tubules are so pronounced that the structures are no longer distinguishable in the homogeneous necrotic masses.

Ureters, Bladder, and Urethra.—In the few cases examined the walls of the ureters, bladder, and urethra showed lesions of the blood vessels, perivascular hemorrhages, and accumulations of round cells similar to those observed in the kidneys and in the lymph nodes.

C. Digestive Organs

1. Liver

In twenty-three cases examined fifteen showed vascular lesions some of which were associated with hemorrhages. Although these hemorrhages were noticed especially around the capillaries the structure of the liver frequently makes it difficult to define the boundaries of hemorrhagic areas or to associate them with definite blood vessel lesions. In addition, the necrotic process in the blood vessel walls extends into the surrounding structures including the parenchymatous tissue and produces lesions very similar to those found in the other organs. In only one case was the necrosis found to extend from the central vein to include up to one-half of the affected lobule (Fig. 6).

In a number of cases infiltration of the interlobular connective tissue with mononuclear cells or connective tissue proliferation was observed, especially around certain bile ducts and blood vessels, as described by Eberbeck (11). Their occurrence is not constant enough to indicate the action of the hog cholera virus in this organ.

2. *Intestinal Tract*

The intestinal tract was studied only in the early stages of the disease process and the same blood vessel lesions were found in the mucosa and, rarely, in the submucosa of the large intestine. Hemorrhages associated with them are as frequently observed as in other organs but foci of necrosis are seldom seen in the early stages of the disease. Hemorrhages are also found around blood vessels and capillaries that show no visible changes. Frequently the capillaries between the glands in the mucosa are enormously dilated and filled with red blood corpuscles. In addition to these lesions there are slight degenerative changes in the epithelial cells, slight edema in the connective tissue, and frequently an accumulation of round cells around the blood vessels in the mucosa, less frequently in the submucosa. In a few cases leucocytes are present in great numbers in the ducts of the glands. Necrosis which sometimes is present extends from changed blood vessels and seems to lead to the formation of ulcers. The study of typical larger ulcers in the intestinal tract is not included in this work.

D. Remaining Organs

The remaining organs, notably the lungs, heart muscle, endocrine glands, and skin, were not included in routine examination because in general they are not the site of typical lesions in this disease. However, in a number of cases in which these organs were studied the same blood vessel lesions were found. Hemorrhages in the skin are not always associated with visible blood vessel lesions. Bronchopneumonic areas in the lungs were seldom present in our material.

DISCUSSION

From this study it is evident that the blood vessel lesions described in the previous paper of this series are responsible in great part for the characteristic pathological picture of this disease. Not only the hemorrhages but also the foci of necrosis in the various organs and the anemic infarcts in the spleen find a logical explanation on this basis. The various organic lesions described in this paper are essentially the result of the more or less pronounced hemorrhages. Frequently inflammatory processes in various degrees, as found in the lymph nodes and kidneys, have complicated the histological picture. It must be admitted, however, that frequently hemorrhages are found around blood vessels that do not show visible changes. On the other hand, even pronounced blood vessel lesions are not always associated with perivascular hemorrhages.

It is important to note that the duration of the disease in general does not determine the type of lesions found in the various organs. Often the course of the disease is so rapid that very severe lesions are found at earlier stages than one would expect. Roehrer (7) believes that the occurrence of splenic infarcts decreases with the length of the disease. However, this observation is not in accordance with our experience. No fundamental difference can be distinguished between the histological lesions in cases of hog cholera produced by virus alone and those complicated by secondarily invading bacteria. While we feel, in agreement with Roehrer (3, 7), that the number and the necrotic character of the lesions in the various organs are not dependent on the presence of secondarily invading bacteria, nevertheless, they are influenced to a certain degree by these organisms. This holds true especially for the lymph nodes, spleen, kidneys, and intestinal tract. In addition, the degree of degenerative changes, especially in the lymph nodes and kidneys, is evidently influenced by compression resulting from large accumulations of red blood corpuscles and round cells. Marked atrophy of the lymphoid tissue in the lymph nodes and spleen frequently seems to be due to the same cause. On the other hand the decrease of lymphoid cells and atrophy of follicles and germinal centers is often present in cases in which hemorrhages are not pronounced. These lesions in the lymph nodes and spleen seem to account for the lymphopenia which is almost always present in virus hog cholera. We are under the impression that the hog cholera virus is able to paralyze the lymphopoiesis in the early stages of the disease, hyperplasia of the lymph nodes and spleen being found very rarely and only in the early stages.

In connection with postmortem diagnosis, the following new observations in the histopathology of acute hog cholera demand consideration: first, the hog cholera encephalitis (see Paper I of this series), and second, the vascular lesions and the lesions in the various organs resulting from them. Waldmann, Roehrer, and Eberbeck (12) emphasize the presence of the encephalitis. We believe, however, that the diagnostic value of hog cholera encephalitis is a limited one because it occurs in only 60 to 80 per cent of the cases and because it is still questionable whether it is strictly specific for this disease. As we have shown, there is the possibility of confusing hog cholera encephalitis and meningitis with the brain lesions occurring in swine influenza and with similar inflammatory processes of unknown nature as described by Ehrlich (13), Fekete (14), Doyle (15), Bendinger (16), and others. According to our present experience, the diagnosis of hog cholera cannot be based on the presence of an encephalitis alone. We feel that the vascular lesions and the changes resulting from them (especially in the lymph nodes, kidneys, and spleen) are far more important since they are almost invariably present. When present the splenic infarcts are of diagnostic value; but they are found in only 40 to 60 per cent of the cases.

In our work the histology in the various organs was very helpful in establishing a diagnosis in a number of cases in which the nature of the disease was doubtful from the gross anatomical lesions. Further investigation must show whether or not these changes can be relied upon in making a diagnosis of hog cholera.

SUMMARY AND CONCLUSIONS

1. The hemorrhages, foci of necrosis, and anemic infarcts met with in the various organs in virus hog cholera, result primarily from the vascular lesions described in a previous paper of this series. Although they are not dependent on the presence of secondarily invading bacteria, their severity is influenced by these organisms.

2. The lesions in the lymph nodes, spleen, kidneys, and central nervous system seem to be of special diagnostic value in questionable cases of hog cholera. The presence of an encephalitis alone does not justify the diagnosis of hog cholera because in the central nervous system of swine similar inflammatory lesions occur in other diseases.

REFERENCES

1. Bel, M. P. A., *Rev. méd. vét.*, 1927, **103**, 168.
2. Nieberle, K., *Berl. tierärztl. Woch.*, 1930, **46**, 717.
3. Roehrer, H., *Arch. Tierheilk.*, 1930–31, **62**, 345, 439.
4. Richter, F., *Arch. mikr. Anat.*, 1902, **60**, 469.
5. Trautmann, A., *Z. Anat. u. Entwcklngsgesch.*, 1926, **78**, 733.

6. David, W., and Schwarz, M., *Arch. Tierheilk.*, 1930–31, **62**, 315.
7. Roehrer, H., *Arch. Tierheilk.*, 1931–32, **64**, 125.
8. Proescher, F., and Seil, H. A., *J. Am. Vet. Med., Assn.*, 1917, **4**, 609.
9. Proescher, F., and Hoffmann, H. A., *J. Comp. Path. and Therap.*, 1924, **37**, 73.
10. Lüttschwager, *Arch. Tierheilk.*, 1931–32, **64**, 236.
11. Eberbeck, E., *Arch. Tierheilk.*, 1931, **63**, 371.
12. Waldmann, Roehrer, and Eberbeck, E., *Arch. Tierheilk.*, 1931–32, **64**, 83.
13. Ehrlich, H., *Tierärztl. Rundschau*, 1926, 276.
14. Fekete, L., *Z. Infectionskrankh . . . Haustiere*, 1928, **32**, 63.
15. Doyle, L. P., *J. Am. Vet. Med. Assn.*, 1929, **28**, 342.
16. Bendinger, G., *Tierärztl. Rundschau* 1932, 137.

EXPLANATION OF PLATES

Plate 23

Fig. 1. Necrotic blood vessels and large necrotic areas in the cell-poor substance of a lymph node. The sinus and peritrabecular tissue is not affected by the necrosis. Hematoxylin-eosin. × 220.

Fig. 2. Necrotic marginal spleen infarct with peripheral hemorrhagic zone. Atrophy of lymphoid tissue in the other parts of the spleen. Hematoxylin-eosin. × 12.

Plate 24

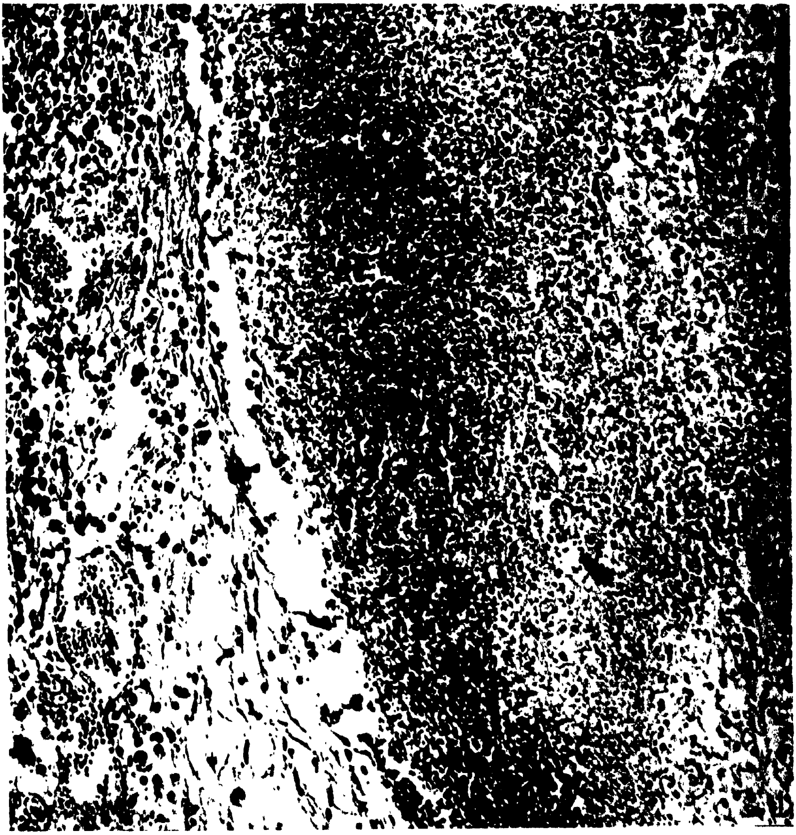
Fig. 3. Apex of necrotic spleen infarct with occluded lumen of a small artery. Hematoxylin-eosin. × 245.

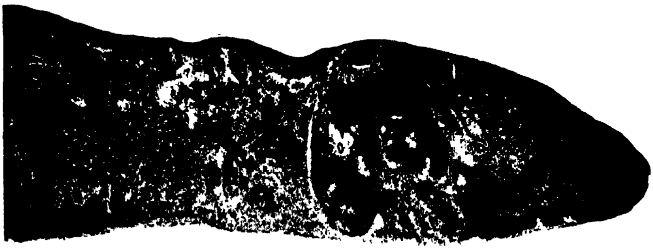
Fig. 4. Hemorrhages in a renal glomeruli and in the interstitial tissues, and degenerative changes of the tubules. Hematoxylin-eosin. × 220.

Plate 25

Fig. 5. Perivascular infiltration with macrophages and lymphocytes in the kidney. Hematoxylin-eosin. × 260.

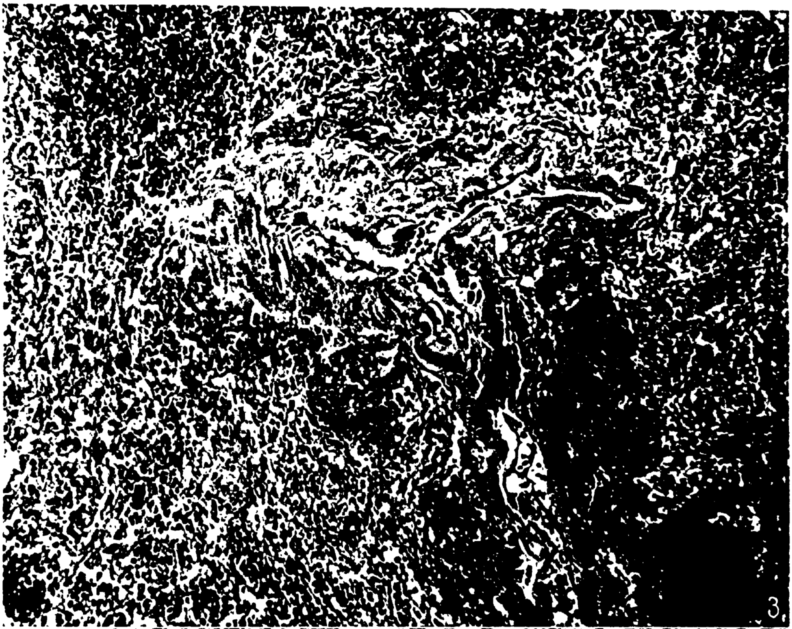
Fig. 6. Extensive necrosis in the neighborhood of a central vein in the liver. Hematoxylin-eosin. × 300.



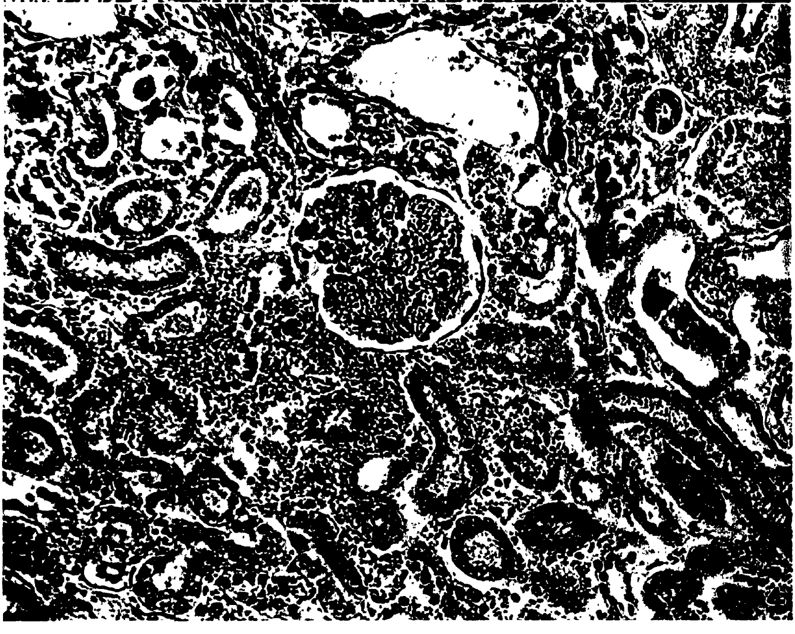


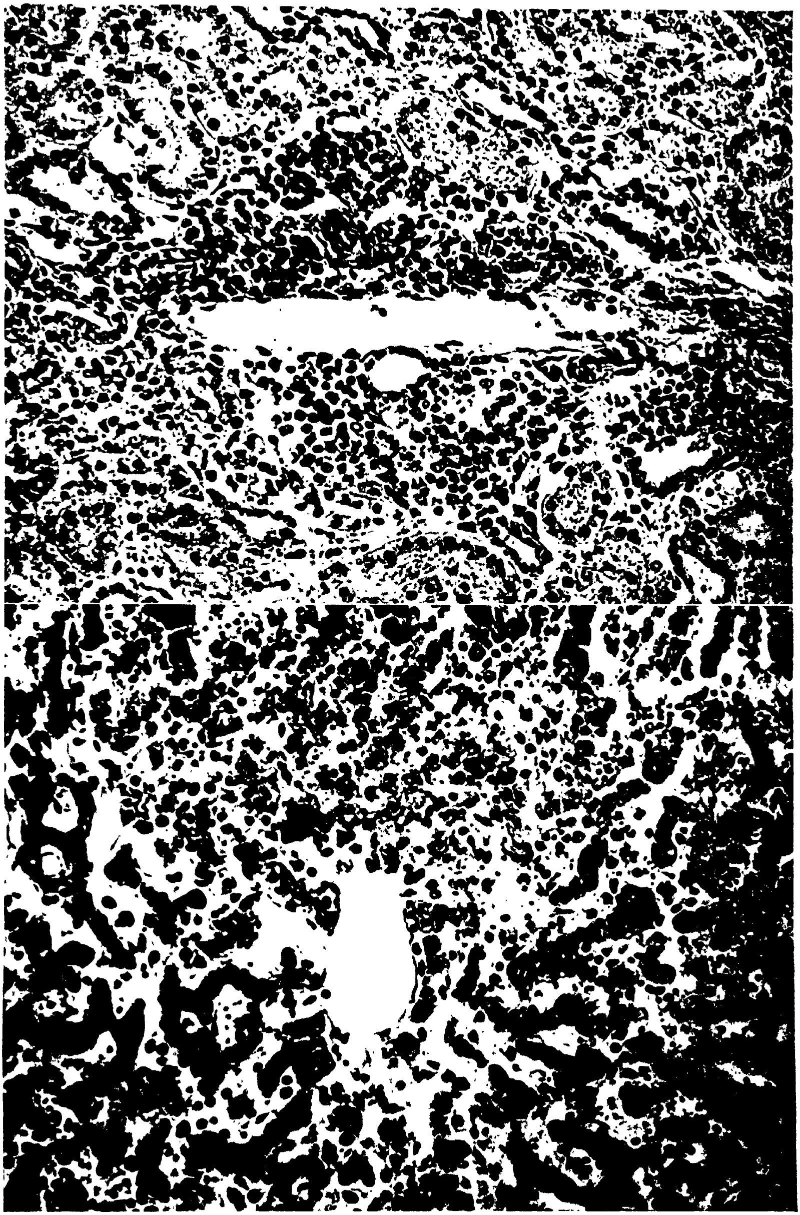
2

(Seifried and Cain: Histological studies on hog cholera. III)



3





(Seifried and Cain: Histological studies on hog cholera. III)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1932,
Vol. 56, No. 4, pp. 575–585]

STUDIES ON IMMUNITY TO SWINE INFLUENZA

BY RICHARD E. SHOPE, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 20, 1932)

INTRODUCTION

It has previously been shown (1) that the bacterium, *H. influenzae suis*, and a filtrable virus are essential to the production of influenza in swine. When administered separately intranasally, *H. influenzae suis* is completely non-pathogenic, while the filtrable virus when similarly introduced induces a very mild and scarcely recognizable illness that has been designated as the "filtrate disease" in distinction from swine influenza which results when virus and organism are administered intranasally together. The present paper describes studies dealing with the immunizing properties of each of the etiological components.

Infectious Materials Used

As in our earlier experiments (1, 2), *H. influenzae suis* was grown upon plain agar slants to the condensation water of which had been added approximately 0.75 cc. of sterile defibrinated horse blood. On such media the growth of the organisms was limited largely to the bloody fluid at the base of the slant and a narrow zone of agar immediately above. A stock culture isolated in December, 1928, and designated as 451, has been used in all experiments recorded in this paper. Only the bloody condensation fluid of cultures was used in inoculating the swine in the experiments to be reported.

The swine influenza virus used was either a Berkefeld N or a Berkefeld V filtrate of lung, bronchial lymph nodes, and bronchial exudate from a fresh case of the disease. Animals to be used as a source of virus were killed on the 3rd or 4th day of their illness. Pathological material to be filtered was minced with scissors and ground fine in a mortar with sterile sand. A suspension of approximately 5 per cent was made in infusion broth, pH 7.3, shaken by hand for 10 minutes, and then centrifuged. The supernatant fluid was removed and filtered through a Berkefeld candle, and the resulting filtrate served as a source of virus.

In testing swine for immunity induced by previous inoculations of virus or

TABLE I

The Effect of H. influenzae suis and Swine Influenza Virus Administered Separately upon the Subsequent Susceptibility of Swine to Influenza

Swine No.	Weight at beginning of experiment	Preliminary treatment		Inoculation to test immunity			Remarks
		Inoculated intranasally with	Result	Length of time after preliminary inoculation or exposure	Inoculated intranasally with	Result	
	lbs.			*days*			
922	19	2 cc. 24 hr. culture HIS* + 8 cc. infusion broth	No illness	18	5 cc. 10% suspension dried and glycerolated influenza virus + 0.2 cc. 48 hr. culture HIS	Swine influenza	No immunity
919	15	8 cc. Berkefeld N filtrate of influenza virus	Filtrate disease	18	" "	No illness	Immune
920	13	" "	" "	18	" "	" "	"
921	17	" "	" "	18	" "	" "	"
923	16	2 cc. 24 hr. culture HIS + 8 cc. Berkefeld N filtrate of influenza virus	Swine influenza				To control effectiveness of organism and virus in inducing swine influenza
959	21	Control			5 cc. 10% suspension dried and glycerolated influenza virus + 0.2 cc. 48 hr. culture HIS	Swine influenza	
1067	45	0.5 cc. 72 hr. culture HIS + 9 cc. defibrinated swine blood	No illness	11	9 cc. light suspension dried and glycerolated influenza virus + 1 cc. 40 hr. culture HIS	" "	No immunity
1125	26	Control			" "	" "	

1155	50	1 cc. 48 hr. culture HIS + 9 cc. physiological saline	No illness	12	14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture HIS	Swine influenza	No immunity
1156	56	Control			" "	" "	
1077	21	2 cc. Berkefeld N filtrate of influenza virus	Filtrate disease	31	10 cc. suspension lung, lymph nodes, and bronchial exudate from influenza-sick swine	No illness	Immune
1082	23	Infected by pen exposure to filtrate disease	" "	24	" "	" "	"
1060	45	Control			" "	Swine influenza	
897	17	Infected by pen exposure to filtrate disease	Filtrate disease	42	8 cc. suspension lung, lymph nodes, and bronchial exudate from influenza-sick swine	No illness	Immune
951	16	Control			" "	Swine influenza	
1130	29	Infected by pen exposure to filtrate disease	Filtrate disease	10	4.5 cc. suspension glycerolated influenza virus + 0.5 cc. 48 hr. culture HIS	No illness	Immune
1136	35	Control			" "	Swine influenza	
1070	59	5.5 cc. Berkefeld V filtrate of influenza virus	Filtrate disease	11	5 cc. light suspension dried and glycerolated influenza virus + 0.5 cc. 48 hr. culture HIS + 10 cc. physiological saline	No illness	Immune
1135	40	Control			" "	Swine influenza	

---

* HIS = *H. influenzae suis.*

organism, where fresh infectious material was not available at the time, glycerolated and dried materials were used. It has previously been shown (1) that the swine influenza virus can be stored for at least 41 days in 50 per cent glycerol or 54 days when frozen and dried by Swift's method (3). *H. influenzae suis*, however, does not survive glycerolation and its survival in dried material is irregular. Therefore in the experiments recorded in this paper in which glycerolated or dried virus was used, small amounts of cultures of *H. influenzae suis* have been added to the suspensions of virus just before using.

The Effect of H. influenzae suis and Swine Influenza Virus, Administered Separately, upon the Susceptibility of Swine to Influenza

As shown in Table I, three swine (922, 1067, 1155) which were inoculated intranasally with *H. influenzae suis* showed no evidence of illness following the inoculation and also were fully susceptible to swine influenza when tested later by intranasal inoculation with suspensions containing swine influenza virus and *H. influenzae suis.*

Five swine (919, 920, 921, 1070, 1077) were inoculated intranasally with Berkefeld filtrates containing swine influenza virus and all developed the mild filtrate disease. When inoculated later with the mixture of virus and organism they were found to be completely immune to swine influenza.

There was a possibility that dissolved products of *H. influenzae suis*, which could conceivably be present in infectious Berkefeld filtrates, might account, at least partially, for this development of immunity in filtrate-infected swine. To test this possibility three other swine (897, 1082, 1130) were infected with the filtrate disease by placing them in the same pens with animals inoculated with infectious Berkefeld filtrates. By this method of infection they received virus free from any trace of *H. influenzae suis* protein, and like the swine infected by intranasal inoculation, they were found subsequently to be immune to swine influenza.

The experiments recorded in Table I indicate that an attack of the filtrate disease, whether induced by intranasal inoculation with filtered virus or by exposure, confers an active immunity to swine influenza as induced by the concerted action of *H. influenzae suis* and the filtrable virus of swine influenza. The administration intranasally of *H. influenzae suis* alone induces no demonstrable immunity to swine influenza. These data indicate that the filtrable virus is of primary etiological significance and that *H. influenzae suis* plays only a secondary and contributory rôle in the clinical entity known as swine influenza.

Neutralization of the Swine Influenza Etiological Complex by Serum of Animals Convalescent from the Filtrate Disease

It has been previously shown (1) that the blood serum of a hog convalescent from swine influenza when mixed with an infectious suspension and administered intranasally to a susceptible animal was capable of preventing infection. It seemed of interest, therefore, to determine whether both of the etiological components were essential to the generation of this neutralizing property of convalescent serum, or whether, as in the active immunization just discussed, the filtrable virus alone was sufficient.

TABLE II

Neutralization of the Swine Influenza Etiological Complex by Serum of Animals Convalescent from the Filtrate Disease

Swine No.	Weight	Inoculated with: Infectious suspension	Inoculated with: Filtrate disease convalescent serum: Source	Inoculated with: Filtrate disease convalescent serum: Amount	Result	Remarks
	lbs.			*cc.*		
1128	36	5 cc. light suspension dried and glycerolated influenza virus + 0.5 cc. 48 hr. culture HIS	Swine 1077 and 1082 pooled	10	No illness	Found subsequently to be immune
1132	29	" "	" "	10	" "	Autopsy negative
1135 (control)	40	" "	10 cc. physiological saline		Swine influenza	Autopsy typical of swine influenza

Filtrate disease convalescent serum was obtained by bleeding two hogs, from the tail, 24 and 31 days after their infection with the swine influenza virus. These sera were freed from bacteria by Seitz filtration and then combined in equal quantities. The procedure was as follows: To 5 cc. of a light suspension of dried and glycerolated swine influenza virus was added 0.5 cc. of the bloody condensation fluid of a 48 hour culture of *H. influenzae suis.* 10 cc. of the filtrate disease convalescent serum was added to this mixture, and after standing at room temperature for 1 hour, it was injected intranasally into the test swine, 15.5 cc. into each animal. The control animal received the same amount of infectious suspension to which 10 cc. of sterile physiological saline had been added. The results are recorded in Table II.

The potency of the infectious suspension was evidenced by the clinical and autopsy picture presented by the control. The neutralization was apparently complete for Swine 1132 which developed no clinical evidence, and, when autopsied on the 7th day after inoculation, showed no pathological evidence of swine influenza. Swine 1128 also at no time appeared ill but on the 3rd day after inoculation it had a temperature of 40°C. This is the lower limit of what we have considered a fever temperature in swine. 2 weeks after its initial inoculation it was found to be immune to swine influenza. It thus seems likely that in this animal the neutralization was not quite complete, otherwise the animal would not only have shown no illness but have remained fully susceptible to infection with swine influenza. A smaller dose of infectious suspension or a larger dose of convalescent serum would probably have resulted in a mixture as neutral for Swine 1128 as the one employed was for Swine 1132.

As in the experiments dealing with the immunity to swine influenza conferred by an attack of the filtrate disease, the neutralization of infectious suspensions, capable of inducing characteristic swine influenza, by serum from swine convalescent from the filtrate disease again indicates the primary etiological significance of the filtrable virus and the secondary contributory rôle of *H. influenzae suis.*

Failure of the Swine Influenza Virus to Induce Illness when Administered Intramuscularly

In these experiments only glycerolated swine influenza virus was used. The cultures of *H. influenzae suis* were of the type used in the experiments previously described in this paper. Glycerolated virus was prepared as follows:

Portions of atelectatic or pneumonic lung of approximately hickory nut size and pieces of the edematous bronchial lymph nodes of somewhat smaller size were taken from swine infected with influenza that had been killed on the 3rd day of their illness. These pieces of tissue were placed in 50 per cent glycerol-physiological salt solution and stored at refrigerator temperature for at least 6 days before use in these experiments. To prepare infectious suspensions from tissues stored in this way, pieces were cut with sterile scissors from the stored material. These were washed in three changes of sterile physiological saline and then ground in a mortar with sand and physiological saline to make approximately a 5 per cent suspension. Such suspensions were allowed to stand undisturbed for a few minutes and the supernatant fluid, when decanted, served as the infectious suspension.

Six swine were inoculated intramuscularly with glycerolated virus. Four of these animals were converted artificially into carriers of *H. influenzae suis* by intranasal inoculation with cultures of this organism. Experiments, which will be reported in detail later, demonstrated that swine receiving *H. influenzae suis* in this way carry the organisms in their respiratory tracts for at least 3 days. The organisms thus carried, while innocuous in themselves, maintain their full potential pathogenicity because when virus alone is administered to such animals swine influenza instead of the filtrate disease results. A carrier state was established in the four above mentioned swine because, should the virus have proven pathogenic when administered intramuscularly, swine influenza as induced by organism and virus together would have been easier to recognize than the filtrate disease induced by the virus alone. In this way the presence of *H. influenzae suis* in the respiratory tract served as an indicator for the invasion of the respiratory tract by swine influenza virus. The data for the six swine inoculated intramuscularly with glycerolated virus together with those for the control swine receiving the virus intranasally are recorded in the first four columns in Table III. It is there shown that while the three controls which received glycerolated influenza virus and *H. influenzae suis* intranasally all developed swine influenza, none of the six swine inoculated with the virus intramuscularly developed any recognizable illness in spite of the fact that four of them were carrying *H. influenzae suis* in their respiratory tracts. One of these animals, Swine 1146, killed 4 days after inoculation, was completely negative at autopsy for any pathology of swine influenza. Of the remaining five, saved to test for immunity, three were given two subsequent intramuscular inoculations of glycerolated influenza virus and showed no reaction or evidence of illness following either of these.

The above data, summarized in the left portion of Table III, indicate that swine influenza virus given intramuscularly is incapable of inducing filtrate disease, or swine influenza, in animals converted artificially into carriers of *H. influenzae suis*. The suggestion derivable from these experiments is that the swine influenza virus is effective in inducing disease only when introduced directly into the respiratory tract, and in this respect is similar to certain other viruses, some of which regularly infect only when introduced into the epidermis (dermatotropic viruses) and others only when introduced directly into nervous tissue (neurotropic viruses).

TABLE III

The Effect of Intramuscular Injection of Swine Influenza Virus

Swine No.	Weight	Inoculation and route	Result	Inoculation to test immunity			Remarks
				Length of time after last inoculation	Inoculated intranasally with	Result	
	lbs.			*days*			
1146	51	10 cc. glycerolated influenza virus i.m.* and 0.5 cc. 40 hr. culture HIS in 10 cc. physiological saline i.n.	No illness				Autopsy negative
1147	61	10 cc. glycerolated influenza virus i.m. and 2 similar subsequent inoculations controlled by Swine 1154	" "	14	14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture HIS	No illness	Immune
1148	63	10 cc. glycerolated influenza virus + 0.5 cc. 40 hr. culture HIS i.n.	Swine influenza				Control for infectivity of virus used on Swine 1146 and 1147 when administered i.n.
1149	77	0.5 cc. 48 hr. culture HIS + 4.5 cc. physiological saline i.n., 10 cc. suspension glycerolated influenza virus i.m., and 2 similar subsequent inoculations with glycerolated influenza virus i.m.	No illness	14	14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture HIS	No illness	Immune
1150	73	" "	" "	14	" "	" "	"

1154	40	10 cc. suspension glycerolated influenza virus + 0.75 cc. 48 hr. culture HIS i.n.	Swine influenza				Control for infectivity of virus used on Swine 1149 and 1150 when administered i.n.
1156	56				14 cc. light suspension dried and glycerolated influenza virus + 1 cc. 24 hr. culture HIS	Swine influenza	Control for immunity shown by Swine 1147, 1149, and 1150
1088	79	0.5 cc. 44 hr. culture HIS + 10 cc. physiological saline i.n., and 10 cc. suspension glycerolated influenza virus i.m.	No illness	15	10 cc. suspension dried and glycerolated influenza virus + 0.5 cc. 44 hr. culture HIS	No illness	Immune
1100	57	10 cc. suspension glycerolated influenza virus i.m.	" "	15	" "	" "	"
1172	33	0.5 cc. 44 hr. culture HIS + 10 cc. suspension glycerolated influenza virus i.n.	Swine influenza				Control for infectivity of virus used on Swine 1088 and 1100 when administered i.n.
1175	19				10 cc. suspension dried and glycerolated influenza virus + 0.5 cc. 44 hr. culture HIS	Swine influenza	Control for immunity shown by Swine 1088 and 1100

* i.m. = intramuscularly; i.n. = intranasally. HIS = *H. influenzae suis.*

Immunity Following the Intramuscular Administration of Swine Influenza Virus

The five animals mentioned above, which were saved to test for immunity, were inoculated intranasally with *H. influenzae suis* mixed with dried and glycerolated swine influenza virus. Swine 1147, 1149, and 1150 had received three intramuscular injections of glycerolated virus prior to their test for immunity while Swine 1088 and 1100 had received only one. All five were found to be completely immune to infection with material which in control swine induced clinically and pathologically characteristic swine influenza.

These experiments are summarized in the right portion of Table III and indicate that swine influenza virus given intramuscularly immunizes hogs against swine influenza without inducing any evidence of illness. So far as can be judged, a single intramuscular injection of glycerolated swine influenza virus confers just as satisfactory an immunity as do three injections.

It is not believed that intranasal inoculation with *H. influenzae suis* contributed to the immunization, in view of the fact that immunity developed whether it was administered or not. Swine 1147 and 1100 which received no *H. influenzae suis* developed as satisfactory an immunity as did the three animals which received the organism intranasally.

DISCUSSION

Evidence derived from the experiments reported in this paper indicates that, of the two components essential to the production of influenza in swine, the filtrable virus is of primary importance while *H. influenzae suis* plays only a secondary or contributory rôle. *H. influenzae suis* administered alone intranasally to swine induced neither illness, as had previously been established (2), nor immunity to swine influenza. The filtrable virus of swine influenza, on the other hand, while capable alone of inducing only the mild filtrate disease, established a solid immunity to swine influenza as induced by the mixture of virus and organism. These findings are supported by the observation that convalescent serum from swine that had suffered only the filtrate disease was capable of neutralizing the combined etiological complex of organism and virus. These results accord with the expected action of two agents of unequal etiological importance but both essential for the production of a disease.

The swine influenza virus showed a certain tissue specificity in that it was found to be incapable of inducing illness when administered intramuscularly to swine although it was uniformly infective when introduced into the respiratory tract. This fact suggests that the swine influenza virus bears a relationship to tissues of the respiratory tract like that of dermatotropic viruses to the skin or of neurotropic viruses to the central nervous system.

It was furthermore of interest, and perhaps of practical value, to note that swine inoculated intramuscularly with the swine influenza virus, while failing to become ill, nevertheless developed an immunity to swine influenza. Under the conditions of laboratory experimentation, intramuscular inoculation of swine with glycerolated swine influenza virus consituted a safe and satisfactory method of immunization. It is possible that this observation can be applied in developing a method of immunization against the disease for use in the field.

SUMMARY

Of the two etiological components of swine influenza, only the filtrable virus possessed immunizing properties. *H. influenzae suis*, while essential to the production of the disease, played only a secondary and contributory rôle and, alone, conferred no immunity. Serum of swine convalescent from the filtrate disease neutralized the swine influenza etiological complex of organism and virus. Intramuscularly administered swine influenza virus was incapable of inducing illness but did render hogs immune to swine influenza. It is suggested that a specific relationship, as regards infectivity, exists between the swine influenza virus and the tissues of the respiratory tract.

BIBLIOGRAPHY

1. Shope, R. E., *J. Exp. Med.*, 1931, **54**, 373.
2. Lewis, P. A., and Shope, R. E., *J. Exp. Med.*, 1931, **54**, 361.
3. Swift, H. F., *J. Exp. Med.*, 1921, **33**, 69.

[Reprinted from THE JOURNAL OF PARASITOLOGY, September, 1932, Vol. 19, No. 1, pp. 54–60]

NOTE ON RE-INFECTION UNDER "NATURAL" CONDITIONS WITH A GUT NEMATODE OF THE RABBIT

BY NORMAN R. STOLL

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

The occurrence in domestic sheep of self-cure and protection against the common stomach worm, *Haemonchus contortus*, under conditions of natural re-infection (Stoll, 1929), led to tests of the same possibility with other worm-host systems. The rabbit appeared to offer the advantage of a useful small-sized host for such study, and wild rabbits of the region as well as some of the domestic stock in the laboratory carried low-grade infections with the small strongyloid *Trichostrongylus calcaratus*, which inhabits the jejunum. This nematode has the characteristic life history of worms of this group: males and females more or less localized during their adult life in a relatively small area of the food tract; no multiplication of the parasites *in vivo* but the extrusion of eggs into the lumen of the tract from whence they escape to the outer world in the feces; then the development in the dung of larval stages, which after an interval of a week or so at summer temperatures become infective, re-enter the host either through the food or skin, and assume final development to adult life within about two weeks in the gut area earlier parasitized by the adult worms which produced them. Such a cycle is characteristic of *H. contortus*, as it is of the hookworms and allied forms.

It seems obvious that if rabbit hosts could be kept under conditions where they would come in contact with soil upon which infected feces would permit larval stages to develop, re-infection would take place, the hosts would be assaulted by gradually increasing numbers of worms and be forced to exhibit their capacity to cope with the parasites.

The requirements of such an experiment appear to be so simple as to make its repetition with various worm-host systems almost an elementary procedure. Experience has taught that practical problems involved in the experimentation frequently render it difficult to resolve however. An early experiment with *T. calcaratus* in domestic rabbits giving results of enough interest to cause its further exploration in some detailed experiments indoors (Sarles, 1932) warrants this preliminary note.

In brief, these results show:

First, that the theoretical demands of the experiment can be met with this worm and host, namely, that clean rabbits will pick up the infection from an area upon which feces containing the eggs of this parasite are deposited, and that reinfection, as judged by egg counts, will take place; and

Second, that while the response of individual hosts is variable, the infection may reach a relatively high level followed in some cases by an indication of a resistance and resultant loss of worms.

These two results are the first such demonstration with this host and parasite.

EXPERIMENTAL

Rabbits from the laboratory stock were utilized. As this stock, particularly at certain seasons, had been known to harbor *T. calcaratus*, precautions were taken to secure animals free from infection as gauged by careful examination of feces by a flotation technique (Stoll, 1930). All these rabbits had coccidia and a few were lightly infected with an oxyurid, *Passalurus ambiguus*. Neither of these parasites appear to have impaired significantly the health of the hosts or the results with Trichostrongylus however.

Preliminary experiments with *T. calcaratus* showed that infection by filariform larvae was possible through both mouth and skin.

Larvae grown in stock cultures of rabbit dung, and isolated by the Baermann apparatus, were used in the following illustrative tests. Rabbit 11, age three months, with feces negative in 5 examinations over an 11 day period, was fed 20 *T. calcaratus* filariform larvae by mouth on May 17, 1928. Five examinations in the next 14 days were negative for ova. On the 16th day there were 26 *T. calcaratus* eggs per gram of dung, 32 per gram on the 19th day, and 6, 12 and 10 per gram on the 25th, 32nd and 39th days after infection. This animal was later used to begin the re-infection experiment.

Rabbit 12, two months old, was used for skin infection. On May 17th, 40 filariform larvae in water were placed on the unabraded skin of the back, after a preliminary wetting of the hair. At 6, 8 and 14 days after infection the animal's feces continued negative; at 16 days 18 *T. calcaratus* eggs per gram were demonstrable. Counts on the 19th and 25th days were respectively 12 and 6 per gram, on the 32nd and 39th days, negative. On the 40th day an additional 15 infective larvae were placed on the unabraded, wet skin of the back. The next examination at 69 days showed a count of 4 *T. calcaratus* eggs per gram, and in 10 examinations about equally spaced over 77 additional days, the animal, kept in a cage designed and believed to exclude re-infection, continued positive averaging 11 eggs per gram (range 4 to 20). On October 12th the animal was killed and 3 adult Trichostrongylus, all females, were recovered.

These two cases are sufficient to show that infections by both mouth and skin are demonstrable, and that even light infections can be shown by the flotation examination of the feces.

The approximate degree of infection with strongyloid worms inhabiting the mammalian gastro-enteric tract is measurable by the number of eggs extruded by way of the feces. This correlation, while not perfect, is sufficiently serviceable to show significant differences in infection levels. The flotation technique used on sheep feces for Haemonchus eggs is highly efficient (Stoll, 1930) routine expectation being that each successive cover after centrifugation from the tube, will remove about nine-tenths of the eggs in the tube. With *T. calcaratus* eggs in rabbit dung each cover removed only about two-thirds of the eggs present. In practice this meant that more covers from a given tube were examined to get adequate counts from rabbit dung than from sheep.

For the experiment itself, the requirement was to place a group of rabbits on soil in a wire-enclosed area from which they could not escape, with one animal infected with *T. calcaratus*, and determine to what degree they would all pick up infection from the area which would be gradually "seeded" by the one infected animal; and thus to determine whether host response to increasing worm increments would be continually in favor of the parasites or of the hosts. This is a type of epidemiological experiment in which all "susceptibles" are equally exposed throughout. The soil enclosure used was 5 x 20 feet, with a cement-floored room into which the rabbits came for food and drink, and for housing at night. The soil area itself, their runway, was clay-loam without vegetation, exposed to both sun and rain, and was occasionally raked up on the surface but not otherwise disturbed. It was shaded in the afternoons. Dung passed in the cement-floored room was swept back onto the soil area daily. Appropriate care was taken that other animals did not have contact with the experimental group, although sparrows were occasionally noted in the area. The rabbits were fed fresh alfalfa hay and grain.

Fecal collections from the animals were obtained by placing them in individual screen-bottom cages for 24 hours each week, during which time they were fed on cabbage leaves, the residue of which could be easily removed. During the experimental period, June to November, only pellet type feces were passed by the animals.

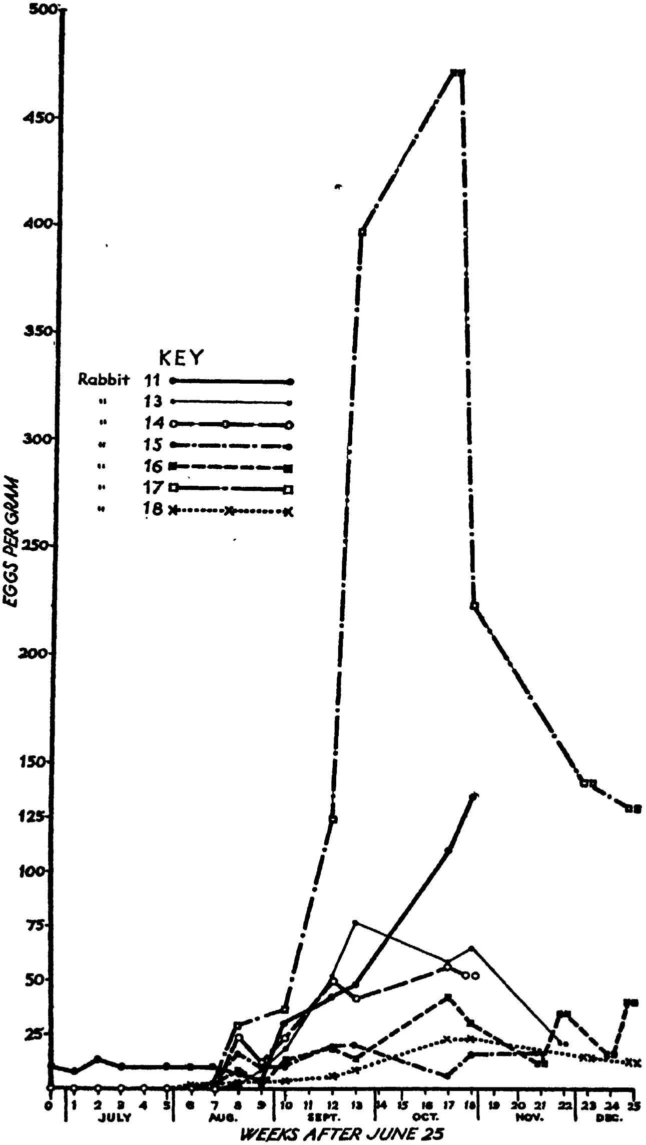
On June 25, Rabbit 11, then about 4 months old, weight 1500 grams, and showing a count of about 10 *T. calcaratus* eggs per gram of feces, was placed in the area, together with 6 clean young rabbits, numbers 13 to 18, litter mates, about two months old. These young rabbits averaged 1215 grams weight each (range 1050 to 1370). The experiment continued until November 15th, by which time temperature conditions were unfavorable for the free-living stages of the worms. An attempt was then made to continue reinfection areas in boxes of soil indoors, but this was not successful, and after a few days was discontinued. As sufficient interval did not elapse for new free-living stages of the worms to develop, it is not considered that the animals were subject to re-infection after coming from the area. In the 20 weeks of the experiment the thrifty condition of the animals was attested by an approximate doubling of the weights of the 6 young animals, to an average of 2280 grams.

As noted, twenty-four hour collections were made each week, although no collections were made the 14th, 15th and 16th weeks (October in part), and those of the 11th week became unusable through inadvertent drying in the laboratory. Figure 1 gives the egg count curves on a per gram basis. As occasional eating of feces by animals in the collection cages was observed, it was not considered feasible to utilize the twenty-four hour total output data as representing a more accurate determination than the eggs per gram. It may be mentioned however that the trend of the eggs per day curves as made up from these collection data differ in no essential respects from those of the eggs per gram shown in Figure 1. Fortunately the high count of Rabbit 17 was determined by a forty-eight hour collection, and is thus unquestionable. It is possible that certain of the animals had higher counts in the 14–16 week interval which were missed by omitted collections, although their possible occurrence does not seriously impair the net result of the experiment. On the 18th week Rabbit 11 was withdrawn from the area for another purpose. Rabbit 14 was killed at the same time and had a post-mortem count of 15 adult *T. calcaratus* (2♂ 13♀). Rabbit 15 was killed the 22nd week and showed 8 adult worms, all females, rabbit 16 the 25th week and showed 6 adults (3♂ and 3♀). The remaining animals were reserved for other uses.

From the experimental data as shown graphically in Figure 1, the following observations are of interest:

1. For several weeks the egg count of the infected Rabbit 11 stayed at a constant level, and the other animals remained negative. This accords with expectation, as it constitutes the period during which the area is becoming seeded with infective stages of *T. calcaratus*, and the required interval between the acquirement of worms by the hosts and the time such worms become functional adults. The consecutive "negatives" indicate also that the rabbits did not ingest eggs from the pellets of Rabbit 11. Inferentially, therefore, later egg counts are from established worms and not by contamination.

2. The gradual development of larger egg counts in all the animals indicated a more or less steady rate of re-infection, which was slow for most of the rabbits. To just what degree this was conditioned by the particular temperature and rainfall of the period need not concern us in this experiment, (although weather conditions in general were held to be favorable for worms), inasmuch as all the animals were kept under identical conditions in one group, and the fact of re-infection is demonstrable. Presumably, and almost surely, re-infection obtained through skin penetration, as the animals were fed indoors on the clean cement floor. So far as the writer is aware, this is the first demonstration of re-infection under natural conditions with a nematode parasite of the rabbit.



TEXT FIGURE 1. Eggs per gram counts of rabbits under natural conditions of re-infection with *Trichostrongylus calcaratus*, a small gut nematode. Circles on the baseline indicate "negatives," *i.e.*, no eggs in the feces. Double spots represent 2-day collection averages. Rabbits 13–18 were litter mates, 2 months old at the beginning of the experiment.

3. There is clearly a difference, as measured by egg counts, in the susceptibility of the individual animals to worms becoming established. This is demonstrably not a function of age as such, with 6 of the animals litter mates. Nor is differential exposure to infective worms a useful assumption, for in addition to the fact that the hosts were all in a group and handled alike, there were no individual differences in behavior noticed which, independently of the egg count evidence, would have suggested differential exposure in the 100 square feet of soil area.

4. Besides the fact of re-infection taking place, and variations in its rate with different hosts, there is in one animal, Rabbit 17, a clear indication of the development of a resistance resulting in a precipitate loss of worms. In less clear-cut fashion a fall in egg count is demonstrable in Rabbit 13. Hint of it may be present in certain of the others, although presumably re-infection conditions were not sufficiently favorable toward the end of the season to force host response, provided one has a right to expect such a response uniformly. The high count of Rabbit 17 reached 475 eggs per gram, and 17,000 per day.

Inasmuch as this experiment was preliminary to a more detailed study of *T. calcaratus* and its rabbit host by Dr. M. P. Sarles in this laboratory, questions of pathogenicity, egg-worm correlations, etc., need not be gone into here, for they form part of the content of his studies.

CONCLUSION

An experiment is reported briefly in which rabbits, exposed under "natural" conditions on soil to re-infection with *Trichostrongylus calcaratus* were shown to develop infections of increasing size, as judged by egg count. Considerable individual host variability in accepting worms was apparent, and in at least one instance a precipitate drop in egg count indicated the development of resistance against the infection, and discharge of worms.

REFERENCES CITED

Sarles, Merritt P. 1932.—Development of an acquired resistance in rabbits by repeated infection with an intestinal nematode, *Trichostrongylus calcaratus* Ransom, 1911. Jour. Parasit., 19: 61–82.

Stoll, Norman R. 1929.—Studies with the strongyloid nematode, *Haemonchus contortus*. I. Acquired resistance of hosts under natural reinfection conditions out-of-doors. Amer. Jour. Hyg., 10: 384–418.

1930.—On methods of counting nematode ova in sheep dung. Parasitology, 22: 116–136.

[Reprinted from THE AMERICAN JOURNAL OF HYGIENE, November, 1932, Vol. 16, No. 3, pp. 783–797]

STUDIES WITH THE STRONGYLOID NEMATODE, HAEMONCHUS CONTORTUS

II. POTENTIAL INFESTATION CURVES UNDER CONDITIONS OF NATURAL REINFECTION

BY NORMAN R. STOLL

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, May 12, 1932)

The opportunity is rarely afforded of being able to define quantitatively the degree of parasitic assault to which hosts are subjected under *natural* conditions of reinfection. When animals are given repeated doses of pathogenic parasites at the experimenter's will, the attack rate to which the hosts would be required to respond when the invaders reach them naturally, may or may not be imitated. In an experiment earlier detailed (1), in which lambs were exposed to natural reinfection with the stomach worm, *Haemonchus contortus*, it has been possible to measure more or less simply the potential infestation to which they were exposed. This "closed universe" of reinfection has thus permitted a graphical expression not only of interest and importance for the particular experiment, but of suggestive value in wider problems.

The reader is referred to the earlier report (1) for details of the experiment upon which the analysis in this paper is based, the essentials of which follow. Twin lambs, numbers 298 and 299 were placed, on an enclosed, nearly level grass plot of 2,800 square feet area. One of them, 299, was given an initial dose of 45 infective *Haemonchus contortus* larvae. The animals were then permitted to pick up what infection they would under natural conditions, following the maturing of this original infection in 299. There was no interference with this process of natural reinfection except that the lambs were moved 3 times, twice because of having cropped off the grass, once because unusual rains put the ground under water. They thus occupied 4 like-sized areas, three of which plots I, II, and III, were contiguous, and one, plot IV, a few hundred feet distant. The soil types, lay of the land, and forage were in each case similar.

The parasite, *Haemonchus contortus*, is looked upon as a typical strongyloid worm, and has a life history that is substantially similar to other strongyloids, of which there are numerous species inhabiting the gastro-intestinal tract of mammals. In the case of *H. contortus* the infective larvae are ingested on the forage, and the worms mature in the fourth, or true stomach, i.e. abomasum, of the sheep. Here the adult worms mate and eggs are shed which leave the host in the dung. During a period of free life in the feces, the embryos pass through changes which result in their reaching an infective stage. At this time the larval worms migrate up grass blades and leaves, where they coil and await ingestion by the host. They withstand some degree of drying in this stage which functions like a spore, to a certain extent.

It is to be remembered that worms of this type, already familiar in the case of hookworms, do not multiply either in or outside the host. One egg passed in the dung can produce but one infective larva, and one infective larva can produce but one adult worm in the host. Their opportunity for survival as a species, and ot causing damage in the host, lies in the fact that the adult female worms individually produce very large numbers of eggs. With a pasture area becoming seeded with enormous numbers of eggs, even a small survival rate during the free living stages permits (as will be demonstrated) a cumulative result in the host which is practically equivalent to multiplication *in vivo*, as obtains with bacteria.

The high egg production of female worms presents the fortunate possibility of demonstrating the presence and approximate number of the parasites in the host by a quantitative determination of the eggs in the dung. The methodology of this determination has been described (1, 2). It is sufficient to note here that this means of following the degree of parasitism in a given host is an unique tool of considerable significance, permitting the helminthologist, as it does, to demonstrate, within reasonable limits, the level of parasitism without dislocating the parasites in the process.

In the experiment described (1), each of the lambs gradually developed relatively large infestations, following which they discharged their worms and became resistant to further infection, although exposed to a prodigious amount of it. This article is concerned with an approximation of the amount of infection they withstood. Thus, while the earlier paper centered primarily on the hosts, this centers primarily on the worms.

Analysis

It is obvious that with a "closed universe" of infection, in which the numbers of eggs leaving the hosts in the feces were determinable, should certain additional items relating to the reinfection process be known or reasonably estimated, the potential degree of reinfestation to which the hosts were subjected can with considerable accuracy be put in the convenient arbitrary statement of the millions of eggs which they would have discharged daily had not self-cure and protection intervened. These items are of an essentially simple nature although requiring certain detailed explanation.

First, by virtue of a known number, 45, of infective larvae given to lamb 299 when the experiment was begun, the eggs expelled later can be stated directly in terms of "per larva ingested." As explained earlier (1, p. 406) this turns out to be about 8,000 eggs per day, with the optimum egg count reached between the 26th and 33rd day following ingestion of the larvae, and remaining at a stable level for a significant period afterward. Thirty days may be accepted, then, as the interval between ingestion and the height of the egg count. It may be noted parenthetically, that this figure does not imply that every original larva administered reached maturity or that this represents a definitive figure for Haemonchus infestations. Neither of these points is determinable from the data nor consequential in the analysis. What it does mean is merely that if lamb 299 at later stages of the experiment did not change its host susceptibility, 8,000 eggs may be taken to represent an ingested larva. A similar assumption is reasonable for the twin lamb 298.

For another point, it needs to be shown that meteorological conditions, especially temperature and moisture, were such as to allow development of the free stages of Haemonchus larvae in an interval and at a rate acceptable to the analysis. The shortest period within which it has been possible to demonstrate infective Haemonchus larvae in dung cultures in this laboratory at room temperature is 5 days. The optimum period is 10 days to 2 weeks. Ransom (3) states, the "final larval stage is reached in three or four days after the eggs have passed out of the body of the host if the temperature remains constantly at about 95°F. At 70°F., six to fourteen days are required, and at 46° to 57°F., averaging about 50°F., three or four weeks are necessary for the eggs to hatch and young worms to develop to the infectious stage. At temperatures below 40°F. . . the eggs remain dormant." Insofar as these results have been tested here they check, adequate moisture conditions of course being implied although below 40°F. the eggs usually perish.

In text figure 1 are given graphically the data on temperature, relative humidity, and rainfall, of the U.S. Weather Bureau at Trenton, N. J., twelve miles distant, for the experimental period, May 20th to October 31st, 1927. (Control readings taken later in a field near the laboratory over a twelve-month period showed that local data follow official Trenton readings closely, the mean monthly temperature being a fraction of a degree less and monthly precipitation half an inch more at the laboratory.) For each week of the experiment are given the minimum and maximum temperatures reached with the weekly average, the daily rainfall in inches, and the resulting relative humidity. The humidity readings were taken at 8:00 a.m., 12:00 m., and 8:00 p.m., so that the maximum and minimum for a given week as well as the average are based on 21 readings. It may be noted that for the entire period of 164 days the mean temperature was 66.1°, daily mean rainfall 0.16 inch, and mean relative humidity 70.7 per cent. These figures substantiate the general impression that the summer of 1927 was to be considered a wet, warm season locally, known to favor parasitic damage in sheep. With weather conditions clearly in the favorable range for the free-living stages of Haemonchus, 10 days from egg deposition to infective stage may be accepted as an average for this interval.

With temperature, humidity, and rainfall conditions recognized as favorable on the whole for the free stages of the parasite, it remains to be shown whether, for rainfall in particular, fluctuations were great enough to give distinctly unfavorable periods. Rain fell on 72 or 44 per cent of the 164 days. The 92 rainless days were divided into 8 single rainless days, 12 periods of 2 consecutive rainless days, 8 of 3, 3 of 4, and 3 of 8, the average period of consecutive rainless days being 2.7. These are obviously short gaps, the 3 periods of 8 days without

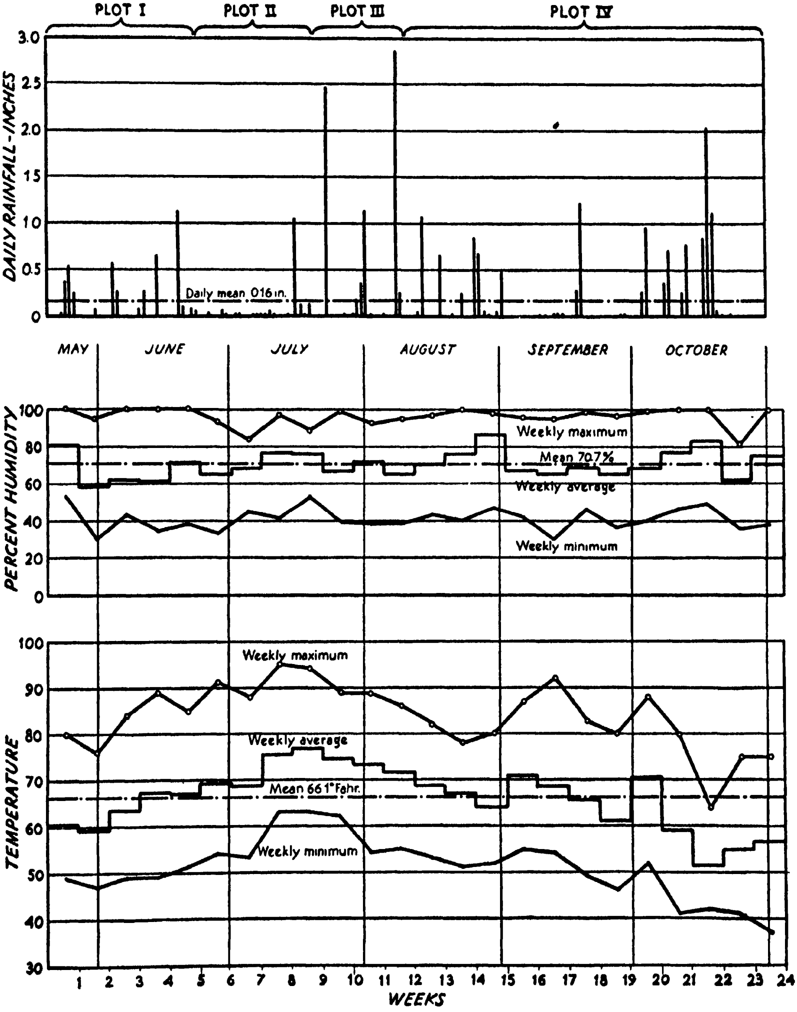


FIG. 1. Temperature, humidity, and rainfall data May 20th to October 31st, 1927. (U. S. Weather Bureau records of Trenton, N. J.) The periods when each of the four pasture plots were occupied are also indicated.

rain coming respectively in the first and last part of September, and the last of October. It must be concluded therefore, that both temperature and rainfall, with the possible exception of the end of the season, were throughout the experiment distinctly favorable for the development of the free-living stages of the nematode.

The analysis to this point has furnished the following information in connection with the "closed universe" of infection:

1. Temperature and rainfall conditions favorable for the development of the parasite to the infective stage;
2. A period of 10 days as an average acceptable interval from egg to infective larva;
3. A period of 30 days after ingestion of the larvae before full egg count of the established parasites;
4. Approximately 8,000 eggs per day at full output as an average figure to represent each larva ingested.

Two further items are required in order to develop the potential infestation curves. One of these is the number of eggs expelled by the animals during the periods of days when the deposited eggs could develop to be infective larvae for the experiment; the other the fractional percentage of eggs eventuating in infective larvae *ingested* by the hosts. In regard to the former, it is obvious from the fact that the degree of infestation in the original experiment was stated in terms of millions of eggs per day passed by the animals, that the total number reaching the areas is determinable by simple arithmetic from the data earlier presented (1, table 2). The cumulative totals are given in table 1 and text figure 2a, in relation to each plot. Eggs expelled totaled 3,600,000 on plot I, 10,300,000 on plot II, 64,700,000 on plot III, and 378,000,000 on plot IV, the huge total during the experiment of slightly less than a half billion eggs (456,600,000). This represents a ten million fold parasitic multiplication, in terms of the original infecting dose of 45 larvae.

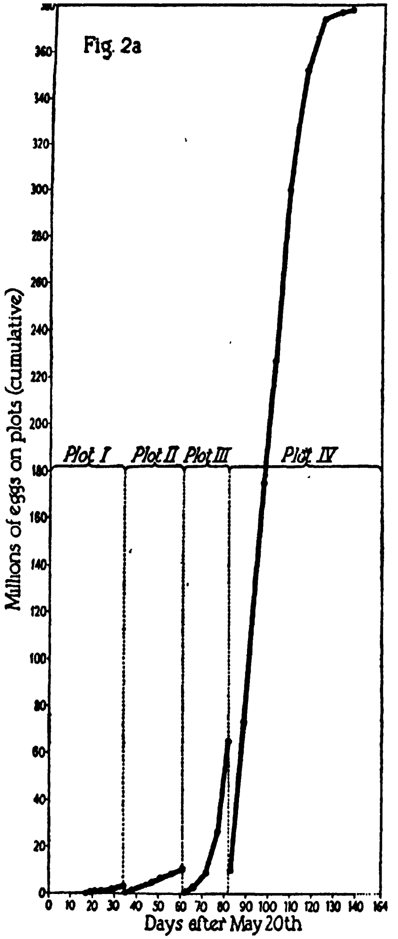
Not all of these eggs had the possibility of producing infective larvae able to reinfect the hosts, however. It is clear that if an average period of 10 days is required for the eggs to develop to infective larvae, then for plots I, II, and III the eggs deposited during the last ten days the animals were on each of these plots, would not have had sufficient time for development to the infective stage before the hosts had gone.

TABLE I

Cumulative Egg Output Totals of Two Lambs with Haemonchus contortus, As Basis for Devising Potential Infestation Curves. (Developed from Raw Data, Table II, Stoll, 1929.) See Text and Figures

	A	B	C	D	E	F	G
	Period of days eggs deposited which later are		Days since beginning of experiment, May 20th	Cumulative totals of eggs deposited on each plot, *in millions*	Potential infestation egg counts, basis $\frac{D}{8,000} \times 8,000$	Potential infestation egg counts, E, additive plot to plot	Day of experiment at 10 + 30 days after C, as time of appearance of potential infestation of column F
	potentially reinfective	*not* potentially reinfective					
Plot I	6		17	0	0	0	—
			19	0.03	0.03	0.03	59
			21	0.16	0.16	0.16	61
			24	0.6	0.6	0.6	64
		10	29	1.8			
			34	3.6			
Plot II	17		35	0.4	0.4	1.0	75
			38	1.5	1.5	2.1	78
			47	4.8	4.8	5.4	87
			51	6.2	6.2	6.8	91
		10	56	8.1			
			61	10.3			
Plot III	11		62	0.4	0.4	7.2	102
			66	2.2	2.2	9.0	106
			72	9.2	9.2	16.0	112
		10	77	26.3			
			82	64.7			
Plot IV	56		83	10	10	26	123
			89	73	73	89	132
			98	175	175	191	138
			103	227	227	243	143
			110	300	300	316	150
			117	352	352	368	157
			124	374	374	390	164
			132	377	377	393	172
			137	378	378	394	177

For a rate of 1/4,000 data of columns E and F would be doubled; for 1/16,000, halved.



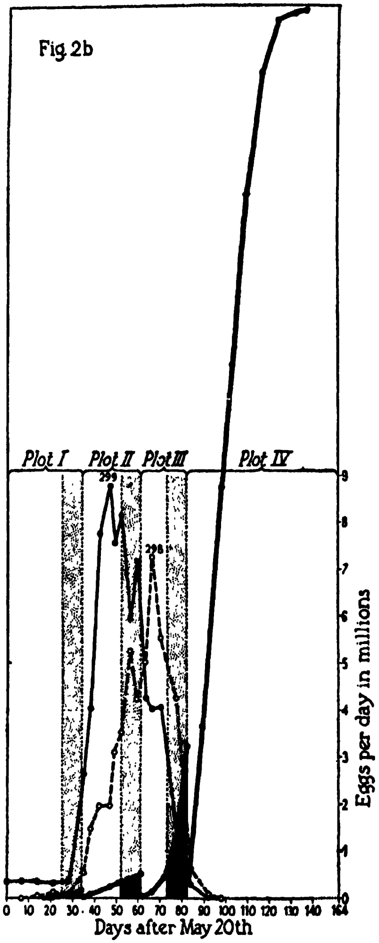


FIG. 2*a*. Cumulative egg deposition in plots I, II, III, IV by two sheep infected with *Haemonchus contortus*. See table I, column D.

FIG. 2*b*. The cumulative egg deposition curves of figure 2*a*, upon which are superposed the actual infestation curves of lambs 298 and 299 *set ahead 40 days* (*i.e. 10 + 30*) *on the time scale*. This permits visualizing the egg depositions responsible for the infestations later demonstrated. For each plot the final period of 10 days is blacked out, as not contributing to reinfection, and these intervals are extended upward to show where they cut the infestation curves.

With these 10-day periods of egg deposition omitted the plot totals for eggs available to develop to infectivity become, for I 600,000, for II 6,200,000, for III 9,200,000, for IV the same as above, or a final total of 394,000,000. As there was no cross drainage between successive plots, the only infection carried from one to the other was that already inside the hosts, where it in turn was cumulative, plot to plot.

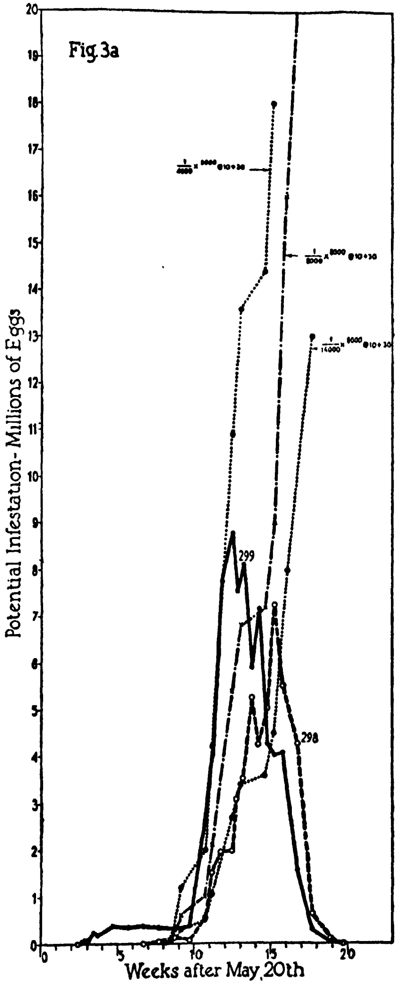
That the interval of 10 days was an acceptable average figure for the eggs to develop to the infective larval stage (and by corollary, permitted the eggs deposited in the final 10 days on each plot to be disregarded as far as reinfection was concerned), is given added support in text figure 2b, where the actual curves of infection of the two animals are superposed on the cumulative egg output curves, *but placed 40 days ahead* so that the infestations accountable in the animals are referable by examination vertically, to the plot and time which furnished the ova eventually resulting in the parasitism. Here it is clear that the first jumps in the egg counts of both animals, which took 299 in fact to its highest point, were from ova deposited and developed on plot II during the period when egg deposition resulted in infective larvae which could be ingested by the hosts there. For that interval on plot II, however, when egg deposition resulted in *no* infective larvae produced in time to be ingested by the hosts, there is shown a lag both in the fall of 299's infection and the rise of 298's. On the other hand, when larvae resulting from eggs deposited in plot III register, 298's infestation curve goes up to a crisis, and 299's continues down. Similarly for plot IV, while during the first week there was a deposition of more eggs by the hosts than on plots I, II, and III combined (a week with 1.11 inches rainfall and 69.7° average temperature) the animals had become so resistant as to fail to show any rise in infection then or thereafter.

The only item now required to develop the potential infestation curves is some sensibly small average percentage of the number of parasites, countable as eggs but infective as 10-day old larvae, which established themselves in the hosts, and registered their full egg count 30 days later. The appropriate small percentage with those picked up from one plot added to those from the succeeding, should develop a curve approximating in its early stages the infection of the animals as expressed by egg count. In trying out various possibilities for this result, a measure was soon encountered with the apparent verisimilitude of an equation developed "backwards" from the rising slope of the infestation curve of lamb 299. Thus if a curve is plotted, using 1 in 8,000 of the eggs expelled by the infested animals as representing the percentage of such eggs which develop through the free-living stages and are ingested, multiplied by 8,000 eggs per day for each ingested larva, the points to be placed at 10 days (interval egg to infective stage) plus 30 days (interval ingested larva to full egg production) after the date of deposition of the originating eggs on the soil, a curve of infestation results, which, as shown in text figure 3a, produces a line that is mean beween the actual infestations of both lambs during the periods of their rising egg counts. Moreover if the percentage is doubled, to become 1 in 4,000, other factors remaining the same, a curve results which quite duplicates the infestation curve of lamb 299 during its rise, and if the rate is halved, to 1 in 16,000 that of lamb 298 is strikingly approximated.

It is probable that the mean line of

$$\text{Curve of infestation} = \frac{1}{\text{8000 eggs deposited}} \times \text{8000 eggs per larva ingested @ } 10 + 30 \text{ days}$$

is as accurate as could be desired for data of this type. Shown to its full extension in text figure 3b, it indicates the enormous disproportion between the resistance of the animals and the potential level of their infestations, a difference between a theoretical 394,000,000 eggs per day output and a negative egg count or nearly so. In other studies it has been impossible to build up infestations of more than 1/7th this total before death occurs to the host. The mean line indicates these animals withstood natural parasitic assault by Haemonchus to the extent of at least 7 multiples of a fatal infection. That we are dealing with reasonable magnitudes here is indicated by the fact that one of the lambs, 299, taken indoors following the outdoor experiment, was given successive doses of infective larvae (1, p. 404 ff.), sufficient to have produced an egg count of 249,600,000 eggs per day, a count equivalent to between 4 and 5 multiples of a fatal infection, and to which it proved resistant 99.96 per cent. There is good reason to believe that such grades of resistance obtain commonly for Haemonchus-resistant sheep on summer pasture.



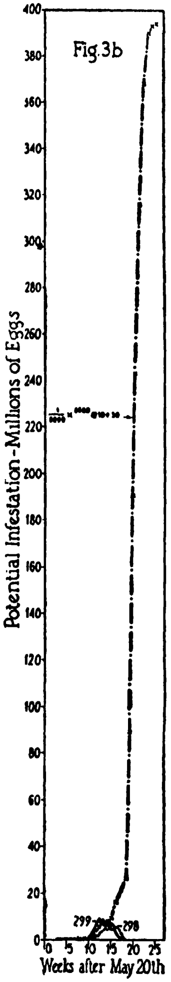


FIG. *3a*. The potential infestation curves plotted in relation to the actual curves of lambs 289 and 299. The data of the mean line $\left(\frac{1}{8,000} \times 8,000 \text{ at } 10 + 30\right)$ are in table I, column F, and are shown extended to completion in figure *3b*. The points on the 1/4,000 and 1/16,000 lines come from the column F values doubled or halved respectively. See text.

FIG. *3b*. The mean line of potential infestation extended to completion.

Turning again to text figure 3a, it is of some interest that the animal, number 299, first infected and which first had a rising egg count, should follow a potential infestation curve (1 in 4000) which is at a rate of 4 times (1 in 16,000) that followed by animal 298. Why did they not behave identically, being twins and exposed similarly? Our observations do not give a conclusive answer. Perhaps they represent merely normal variability, even between close sibs, when exposed to any infection. Perhaps while maintaining practically equivalent weight curves (1, p. 401), 299 may have been a naturally closer grass cropper than twin 298; or, what is more plausible, that the original light infestation given the lamb may have induced more voraciousness or some geophagy (the latter a symptom common in verminous sheep) enabling him to secure during the time he was on plot II that slightly greater ingestion of infested forage which would account for greater increments of worm.

DISCUSSION

In the "closed universe" of infection under study a relatively simple set of conditions is being dealt with: only two hosts, similar sized areas pastured, for known periods, known total of ova deposition on the areas, and known meteorological conditions. The significant factors used to determine the potential infestation curves have considerable reliability within the limits of this "closed universe." Thus, egg outputs of the infected animals, considered in connection with the initiating infection of 45 larvae administered to lamb 299, permit direct determination of the figure of 8,000 eggs per day per larva ingested, after a 30-day interval following ingestion of the larvae. No greater error can be involved in those two factors than may inhere in the original data, as the former are a direct function of the latter. Again, the use of 1 in 4,000, 8,000 or 16,000 of the number of ova finally resulting in ingested infective larvae is the "unknown" factor of the analysis, and, as explained, is that small percentage which employed additively reproduces the actual curves of the infection during the time they are rising. Due to this exceedingly small percentage, little variation in the curves would result if the *mean* value of 10 days from egg to infective larval stage were slightly more or slightly less than 10 days. As a matter of fact, the latter figure not only accords with general knowledge of this form under prevailing temperature conditions, but also with the implications of the supposed actual infestation curves as set ahead in text figure 2b. It is of interest to note that if an interval slightly greater or less than 10 days is used, with slightly different rates of acquirement of the larvae, the rising infection curves of lambs 298 and 299 can also be simulated, although the fit does not appear to be quite so good. Such alternative factors however all eventuate in an enormous disproportion similar to the one illustrated between the potential and actual infestations after the animals became resistant.

While it is possible, even probably, true that no *mean* value for any factor obtains throughout every day and hour of an experiment such as the one under analysis, it must also be possibly, even probably, true that the net effect of the variations in the given factors produces a result similar to an average effect for each factor, provided that a direct comparison exists by which the concept can be tested. This, as already pointed out, is inherent in the analysis, the potential infestation curves simulating the rising portions of the actual infestation curves.

The further possibility that 8,000 eggs per day represents one ingested larva ony when the count is small, with an equivalent number of eggs representing more worms when the count is large (as Hill's data (4) on human hookworm, and Sarles' (5) on dog hookworm imply), would have the tendency to dampen the potential infestation curve as it increased in height. This possibility is biologically admissible, representing the tendency to decreased egg production for the individual female worms under conditions of crowded worm populations. It may already have expressed itself within the range of the actual rising infestation curves of the two hosts. To this extent the "damping" effect, if present, is likely absorbed to a significant degree in the low 1/4,000, 1/8,000, and 1/16,000 rates found applicable. There appears as yet no calculable factor to express the situation further.

Certain additional points of interest emerge from the analysis.

1. It is a striking fact that the percentage of 1 in 4,000, 8,000 or 16,000 eggs deposited on the plots as representing the later infective larvae ingested by the hosts, is an exceedingly small return from the standpoint of the parasites themselves. Indoors, in dung cultures, it is usual to get a yield of about 10 per cent of infective larvae from the

eggs cultured, equivalent to 800 in 8,000, and yields up to 70 per cent are not rare. Of course the rate of 1 in 8,000 depicted represents the survival as against all the forces deleterious to the worms from the time the eggs are expelled, through the developmental stages, through the migration to and waiting on the forage, the cropping of such forage, and passive ingestion by the hosts. The losses working against even such an "efficient" sheep worm as Haemonchus, are thus enormous, for while potentially 1 egg may later represent 1 more adult worm in a susceptible host, actually it appears to take under pasture conditions from 4,000 to 16,000 eggs to establish an additional parasite in an abomasum. The vicissitudes of the parasites in their free life are seen then to be very great indeed, and are to be added to their vicissitudes as a species in the presence of resistant effects producted in hosts whereby the host becomes an untenable habitat after the hardships of the free life are abnormally past.

2. Despite these hardships to which Haemonchus is subject in the free stages as well as the parasitic, it is worth emphasis that an original infecting dose of 45 larvae to lamb 299 in the original experiment resulted eventually in the deposition within four months by only two lambs of the enormous total of nearly half a billion eggs on the plots. Reduced to a simpler form this is an amost unbelievable figure, for if this deposition rate had been constant for the 119 days in which eggs were expelled by the two hosts, a rate of *44 eggs per second* is indicated for this entire period. As a matter of fact, for 35 days of the period it was between 2 and 3 times this rate. Obviously, large numbers of adult female parasites were active to accomplish this, but here is a good example of what is meant by the enormous fecundity of a parasitic nematode like *Haemonchus contortus* attempting to maintain species continuity, for each original ingested larva eventually multiplied itself ten million fold in individual eggs produced over a period of twenty weeks.

3. The fact that the animals in the experiment occupied four successive pasture plots during the experiment, might appear to render an analysis of the type presented rather more difficult than if they had occupied but one plot. More careful consideration indicates, however, certain distinct advantages in their having occupied four plots. The area of each plot at 2,800 square feet, while comfortably large for the

lambs, was sufficiently small to bring about good cropping of the grass and thus more certain reinfection. Moreover the shift from plot to plot later emphasized the great differences which developed in the susceptibility of the hosts, as determined by the comparison between the number of eggs expelled, and the amount of infestation resulting therefrom. On plot I, eggs were deposited by lamb 299 to the number of 600,000 in 6 days (omitting those of the last 10 days), and this deposition on the soil of an average of 100,000 daily produced a demonstrable infection in clean lamb 298. On plot II eggs were deposited (again by lamb 299 alone) to the number of 6,200,000 over a period of 17 days (omitting the last 10 days), and this deposition on the soil of an average of 370,000 daily was the demonstrable cause of lamb 299 receiving an infestation that measured up to nearly 9 million eggs daily output, and of lamb 298 to over 3 million. The infective stages of Haemonchus resulting from the eggs deposited have, on these two plots, reinfected essentially susceptible hosts.

A change in the picture occurs for plots III and IV. On plot III eggs were deposited, both hosts participating, to the number of 9,200,000 over a period of 11 days (omitting the last 10 days), and this deposition on the soil of an average of 830,000 daily was the demonstrable cause of lamb 298 receiving an infestation that measured over 7 million eggs daily output, while the now partially resistant lamb 299 dropped to 4 million. And on plot IV, while the lambs deposited 10 million eggs on the soil the first day they were on the plot, a daily rate that was maintained or exceeded for 26 of the next 27 days, and all told in a period of 56 days resulted in the deposition of 378,000,000 eggs, there was not only no increase in the host infestations resulting from larvae growing on this plot, but the animals became and remained entirely resistant. This might suggest that external conditions on plot IV were essentially bad for larvae to develop. Such a conclusion is negatived by three observations; namely, that with the possible exception of 2 gaps of 8 rainless days each during this 56-day period of egg deposition, rainfall and temperature were distinctly favorable for the free-living stages (the third 8-day rainless period came later), that (1, p. 400) infective Haemonchus larvae were demonstrated in the feces after having passed through the resistant animals without parasitizing them, and that in the next season, 1928, this area permitted a fatal infestation of Haemonchus to develop in a susceptible animal.

It is true, of course, that one has in *Haemonchus contortus* and its sheep host almost ideal conditions for reinfection. As Hall's phrase so aptly emphasizes, this host "soils its own table." The fact that the infective larvae reach the host on forage, passively, gives a peculiarly effective persistence to this worm-host system, and readily accounts for the serious pathogenic effects in such sheep as do not change this host susceptibility by developing a resistant state. At the same time it must be true that all hosts which are under conditions of reinfection with parasitic worms are under the necessity of responding to some variant of the type of potential reinfection curve illustrated in figure 3b. Differences are differences in degree, not in fact. Especially does hookworm in man in the tropics suggest itself, where, in the absence of sanitation barefoot natives revisit sites of previous defecation and become reinfected. Why under such conditions do not more cases develop of overwhelming worm burdens with fatal ancylostomiasis or necatoriasis? Is it not easily possible that the generalities of the case require more detailed consideration of the probable intervention of just such a resistant reaction as has been demonstrated for Haemonchus in sheep (1), for *Trichostrongylus calcaratus* in rabbits by Sarles (6), and for *Ancylostoma caninum* in dogs by McCoy (7)?

SUMMARY

Analysis is given of a "closed universe" of infection, in which two lambs were exposed to *Haemonchus contortus* under conditions of natural reinfection. From the data available it has been possible to develop curves of potential infestation, illustrating the enormous disproportion between the presumptive and actual infestations of animals under *natural* conditions of interplay between parasite and host.

BIBLIOGRAPHY

1. Stoll, N. R.
   1929. Amer. Jour. Hyg., *10*, 384.
2. Stoll, N. R.
   1930. Parasitology, *22*, 116.
3. Ransom, B. H.
   1907. Bur. of Animal Industry, U. S. Dept. of Agriculture, Circ. 102, 7 pp.

4. Hill, R. B.
1926. Amer. Jour. Hyg., *6*, July Supplement, 19.
5. Sarles, M. P.
1929. Amer. Jour. Hyg., *10*, 658.
6. Sarles, M. P.
1932. Jour. Parasit., *19*, 62.
7. McCoy, O. R.
1931. Amer. Jour. Hyg., *14*, 268.

[Reprinted from THE JOURNAL OF PARASITOLOGY, September, 1932, Vol. 19, No. 1, pp. 61–82]

DEVELOPMENT OF AN ACQUIRED RESISTANCE IN RABBITS BY REPEATED INFECTION WITH AN INTESTINAL NEMATODE, TRICHOSTRONGYLUS CALCARATUS RANSOM, 1911

BY MERRITT P. SARLES

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

In a short paper dealing with reinfection of the domestic rabbit with a gut nematode, *Trichostrongylus calcaratus*, under natural conditions, Stoll (1932) presented evidence that in some cases at least this host illustrated an effect he had demonstrated earlier (1929) with domestic sheep and a stomach worm, *Haemonchus contortus*; namely, an initial development of parasitism followed by an apparent host response with discharge of worms. The present article deals with a further exploration of this reaction in a small group of rabbits kept under laboratory conditions precluding natural reinfection, but given definite increasing doses of *T. calcaratus* larvae at regular intervals, and followed by frequent counts of the number of parasite eggs in the feces in order to determine the ultimate effect on both hosts and parasites.

Investigation of acquired immunities to parasitic nematodes is comparatively recent. Sandground (1928) found an immunity in dogs and cats to a second infection of *Strongyloides stercoralis*, an intestinal parasite of man, but regarded it as probably due to the fact that the parasite was in an abnormal host. Stoll (1929) emphasized the importance of studying animals exposed to repeated infection since "the usual hosts of nematode parasites are not infected just once in their lives, but receive more or less constant increments of additional parasites, at least seasonally." He demonstrated that sheep exposed to such reinfection with *H. contortus* developed a resistance, or self-cure and protection, and stated that this phenomenon, if of general occurrence among other hosts and parasites, would necessitate a revision of the generally accepted ideas concerning helminthic infections. The advance abstract of the present paper (Sarles, 1930) indicated that the phenomenon was not limited to sheep, and predicted that it could accordingly be expected to occur with other hosts and worms when appropriately studied. This statement has been substantiated by subsequent reports of acquired resistance to parasitic nematodes, many of which may be interpreted on the basis of self-cure and protection.

McCoy (1931a) studying *Ancylostoma caninum* in dogs and cats demonstrated what he interpreted as an "immunity reaction" following repeated infection. Casual evidence of increased resistance in dogs after previous infection with hookworms had been reported earlier by Payne (1923), Herrick (1928), and Sarles (1929), using *A. caninum*, and by Fülleborn (1926), using *Uncinaria stenocephala.*

An acquired resistance in rats following infection with *Nippostrongylus muris* has been reported by Schwartz, Alicata and Lucker (1931), Africa (1931), and Chandler (1932). Rats also acquire a relative immunity following infection with *Trichinella spiralis*, as shown by McCoy (1931b). An acquired resistance in chickens to infection with *Ascaridia lineata* has been reported by Graham, Ackert, and Jones (1932).

Materials and Methods

The parasite used in this study, *Trichostrongylus calcaratus* Ransom (1911) is one of the bursate nematodes with the following life history: entrance into the host as a filariform larva; growth to adult stage in a relatively localized area of the gastro-intestinal tract (in this case principally the jejunum); mating of males and females and production of large numbers of eggs into the lumen of the gut from whence they escape into the feces; embryonation and hatching of the eggs in the dung, and development into the infective larval stage after a week or two under favorable moisture and temperature conditions. Stoll (1932) has shown that infections of *T. calcaratus* may result from the larvae penetrating through the skin, or by ingestion.

The fact should be emphasized that there is no multiplication of the worms within the host. One egg develops into one larva, which after developing to the infective stage may parasitize a host and become one adult male or female worm. Hosts which harbor large numbers of worms have, therefore, necessarily received large numbers of larvae.

Under natural conditions the number of eggs shed by the individual female worms gradually permit an extensive number of infective larvae to develop and reinfect the host. The numerous eggs given off into the gut by the adult female worms permit the experimenter to follow the degree and fate of the infestations by the simple means of egg counting the feces.

Inasmuch as rabbits from our laboratory stock occasionally show light infestations of *T. calcaratus* and *Obeliscoides cuniculi* (a stomach worm), we obtain them when they are weaned at 6 weeks of age, and only use for experimentation those which show no worm eggs in their feces during a subsequent period of at least 3 weeks. Such animals can be reasonably assumed to have never been infected with the two above species. The 7 rabbits reported in this paper showed only coccidial oocysts upon weekly examination of their feces by flotation during the 3 to 6 week period which elapsed from the time that they were weaned until their first infection. They were kept in metal cages throughout the experiment, on one-half inch mesh wire screens fastened over the top of a metal pan. The screens were scrubbed daily, and the rabbits were transferred each week to a clean set of cages which had been sterilized in boiling water. In one rabbit (No. 110) small numbers of the eggs of the rabbit oxyurid, *Passalurus ambiguus*, were noted at two different periods during the experiment. It does not seem probable that the oxyurid or coccidial infection had an appreciable influence on the outcome of the experiment. A control rabbit remained uninfected throughout the experiment indicating that no infections were being picked up from food or in cage transfers.

Screens supporting the rabbits allowed the fecal pellets to fall on moistened paper towels in the 3 inch deep pan below, from which they were collected daily and their consistency noted. The 24 hour specimen was weighed, a portion taken for cultures and the remainder either used immediately for making up egg count flasks or kept under refrigeration until used. Two egg count flasks were made up using 10 grams of pellets to 300 cc. of diluent: a water flask for flotation counts and a sodium hydroxide flask for dilution egg counts. The manipulation and counting of these flasks were in strict accordance with the steps recently outlined and discussed in detail by Stoll (1930). All counts were made by the author, the collection and tubing of specimens being done by an experienced technician under supervision. The one pipette used for all the dilution egg counts, together with all flasks, etc., were calibrated against Bureau of Standards certified pipettes. The flotation method was used to detect the first appearance of eggs in the feces and for counts under 200 eggs per gram, whereas the dilution counting technic was utilized for all larger counts. The flotation counts were carefully established and checked by counting 3 covers from each of 2 tubes, i.e. the eggs from one gram of feces were enumerated. The dilution egg counts may also be regarded as being highly reliable since they were based on the counting of 2 to 9 large drop (0.15 cc.) slides from each flask, with an average of 4.5 slides, utilizing sufficient slides to reach the 5 per cent limit (Stoll, 1930) on 78 of the total series of 91 flasks and the 10 per cent limit on 11 other flasks. It is not feasible to present in this paper the results of a statistical analysis of the egg count data secured beyond the statement that both of the counting methods employed were demonstrably satisfactory for enumerating *T. calcaratus* ova in rabbit dung.

Originally it had been planned to make daily counts on all of the rabbits but this was soon found impracticable, and the number was reduced to 2 or 3 a week, except in the case of Rabbit 103, which was followed daily from the 14th to the 139th day. In the final weeks of the experiment counts were not made oftener than once a week.

The larvae used for the first 2 infections were cultured from one of the animals earlier reported by Stoll (1932). Larvae for later infections were cultured from the feces of the 6 rabbits of the present experiment, and from 4 other originally "clean" rabbits infected from them. High and uniform infectivity of the larvae, and purity of the parasitic species throughout the study were proven by the nature of the infestations produced in 12 other rabbits infected with larvae from the same cultures used in this experiment, and by the recovery of only *T. calcaratus* from these animals at autopsy.

To secure infective larvae, fecal pellets from infected rabbits were left in a Petri dish, moist chamber, or pasteboard carton, at room temperature for 10 days and then isolated in the Baermann apparatus (Cort, et al., 1922). The larvae were then washed several times in tap water and counted under the microscope, using a 40 mm. objective and a 10 × ocular. Doses up to 400 larvae were counted direct, but larger doses were done by dilution, by counting two or more one-half cc. samples from a larval suspension of 10, 25, or 50 cc. The degree of accuracy of these dilution counts was ascertained by the method of Scott (1928) for each of the 3 dilutions that were used. For suspensions of 10 cc., which were used to count doses of 400 to 4,000 larvae, the coefficient of variation of the average of two counts was 12.5 ± 1.2 per cent; on suspensions of 25 cc. and 50 cc., which were used to count doses of 1,000 to 21,000 larvae, the coefficient of variation was 8.5 ± 0.8 per cent.

Mouth infections were given by holding the rabbit's jaws open and pipetting into the back of the mouth a few drops of water containing the larvae. The rabbit swallowed readily in most instances, with only occasional slight loss of fluid. The skin infections were made by clipping the hair as closely as possible over an area 3 to 4 cm. in diameter, placing the larvae on this area in a thin film of water and allowing the water to evaporate slowly (15 to 30 minutes), while the animal was held in one position. Each successive cutaneous infection was made on a previously unused area of the body.

The ages of the rabbits when first infected November 16, 1929, were three months for Rabbits 103 and 104, which were litter mates of the control, and two and one-half months for Rabbits 110, 111, 112 and 113, also litter mates. The second series of infections were given when the animals were 21 weeks older.

The rabbits were kept on a uniform diet consisting of a daily ration of one-half pint grain mixture, one-fourth pint moistened beet pulp, and 4 to 8 ounces of green cabbage, fed in door cups each morning, with water given freely. On this diet, which appeared to be adequate, the rabbits produced formed fecal pellets that crushed easily in the egg count flasks and contained very little coarse vegetable débris. Such dung has been found optimum for convenience and accuracy of egg counting and well suited for the culturing of *T. calcaratus* larvae.

It was planned to build up infestations in the 6 rabbits by giving weekly doses of increasing numbers of larvae; 3 animals to be infected by mouth, and 3 by skin. Partly for convenience, and partly to simulate the assault of an increasing number of parasites, such as hosts might be exposed to when on limited areas out-of-doors, each succeeding dose was to be the sum of all earlier ones. As a matter of fact, the requirements for infective larvae outstripped our ability to culture them at first, when we were dependent upon a single animal for cultures, and also towards the end of the initial series of infections when the practically simultaneous loss of worms by 5 of the 6 rabbits was reflected in a rapidly decreasing yield of larvae from cultures. Consequently it was impossible to maintain the weekly doses during the first two months of the experiment, and some of the final doses were not increased in size to the degree planned. With these exceptions the plan of weekly additive doses was closely followed, as may be seen from Figures 1–3 and 6–8. This method of infection is a process by which, substantially, the experimenter assists in developing, with a parasite which does not naturally multiply in its host, total infestations of a size comparable to what might have been obtained by a parasite multiplying *in vivo*. As it was known that this parasite has the potentiality of being fatal for its host when present in large numbers, such a building up of the worm burden provided a method of forcing a host response.

RESULTS

The course of the infestation produced in each of the 6 rabbits by the doses of larvae which it received are shown in Figures 1–3 and 6–8, which are graphs of the eggs per day counts listed in Tables 1 and 2. (The infections showed the same course in terms of eggs per gram counts as used by Stoll (1932), but the counts were somewhat more variable from day to day.) In these figures the crosses indicate the number of eggs which were passed on each day that a count was made (except in the case of Rabbit 103 where they represent 3-day moving averages of daily counts) while the open circles on the base line show that an examination was negative. At the top of the graphs are listed the number of larvae given at each successive dose. The arrows indicate the size of these doses and when given, and also constitute a theoretical curve of expected egg production based on the number of eggs which appeared following the first dose of larvae. The first dose given to the 3 orally infected rabbits produced average egg counts for the 3rd week after infection of 23, 32 and 28 eggs per day per larva, with a mean of 28, which was the factor used for plotting the dosage arrows for all oral infections. The first dose given the 3 cutaneously infected rabbits yielded for the same period 6, 4, and 0 eggs per day, with a mean of 3.3, which was the factor used for plotting the dosage arrows for all skin infections. As it takes approximately 2 weeks for the larvae to develop into adult worms and produce eggs, and another week for the count to reach its maximum, each arrow represents the increase in the egg count which might be expected during the 3rd week after that infection if the rabbit were as susceptible to all doses as it was to the first one. The factor used for the oral infections is a very

TABLE 1

Eggs Per Day Counts (in Thousands)

Days after first inf.	Rabbits					Days after first inf.	Rabbits					Days after first inf.	Rabbits			
	104	110	111	112	113		104	110	111	112	113		110	111	112	113
3	N	N	N	N	N	44	0.3	3	0.2	1	1	111	—	—	51	N
10	N	N	N	N	N	47	—	4	—	2	—	114	N	0.1	86	N
13	N	0.1	N	N	N	48	—	3	—	3	—	121	1	2	15	N
14	N	0.1	N	N	N	49	—	—	—	5	—	128	8	N	7	N
15	N	0.2	N	0.2	N	50	—	—	—	3	—	135	N	N	11	0.7
16	0.3	0.7	0.1	0.5	N	51	0.3	3	0.5	8	0.4	142	N	N	10	4
17	0.1	1	0.1	1	N	54	0.5	2	0.4	5	0.5	149	N	N	22	4
18	0.2	1	0.3	1	N	58	0.6	4	0.3	4	0.2	156	N	N	9	1
19	0.7	1	0.3	4	N	61	0.2	3	0.3	10	0.3	158	N	N	13	2
20	0.5	2	0.1	2	N	65	0.4	10	0.5	15	0.4	160	N	N	17	0.5
21	0.1	4	0.1	1	N	67	0.5	10	0.9	13	1	163	N	N	7	0.2
22	0.2	2	0.1	0.4	N	69	0.8	13	0.4	20	1	165	N	N	14	N
23	0.3	4	0.6	4	N	72	0.6	23	1	62	2	167	N	N	14	N
24	0.5	3	3	2	N	74	0.5	21	3	81	1	170–1	N	N	7	N
25	0.1	—	0.5	2	N	76	0.4	21	1	50	2	177–8	N	N	16	7
26	0.5	3	0.4	2	0.1	79	2	45	1	69	1	191–2	N	N	17	N
27	0.1	3	1	1	1	81	1	16	1	74	2	212–3	N	0.8	18	7
30	0.4	4	0.4	2	0.1	83	3	22	1	79	7	219	0.1	0.1	—	1
31	0.2	2	0.5	2	0.5	86	7	12	2	5	7	226–7			15	
33	0.1	2	0.6	2	1	88	—	17	—	N	—	254–5			16	
34	0.2	1	0.3	1	0.4	90	9	0.1	2	0.2	10	282			N	
36	0.6	2	0.4	3	0.1	93	24	N	2	0.2	15					
37	0.5	3	1	1	0.7	97	24	—	4	—	18					
40	0.4	5	1	3	0.7	100	10	N	5	1	34					
41	0.4	1	1	2	1	104	2	—	0.1	—	31					
43	0.3	1	1	1	0.4	107	N	N	N	11	15					

N = negative for eggs.

conservative one when compared with that of 43 eggs per day per larva produced by the single infection of 2,500 larvae given to Rabbit 116. Accordingly the failure of the egg counts to increase with the theoretical curve is somewhat minimized.

TABLE 2

Daily Egg Counts on Rabbit 103 (in Thousands)

Days	EPD	Days	EPD	Days	EPD	Days	EPD	Days	EPD
3	N	40	4	68	62	96	63	124	87
10	N	41	1	69	58	97	66	125	90
11–13	N	42	2	70	62	98	34	126	65
14	N	43	3	71	82	99	75	127	100
15	1	44	2	72	132	100	51	128	100
16	1	45	1	73	114	101	92	129	74
17	0.1	46	2	74	109	102	41	130	103
18	1	47	3	75	96	103	21	131	136
19	1	48	1	76	65	104	27	132	69
20	1	49	3	77	96	105	17	133	72
21	1	50	2	78	93	106	44	134	79
22	0.04	51	1	79	94	107	17	135	70
23	3	52	3	80	162	108	53	136	69
24	1	53	3	81	103	109	73	137	226
25	0.1	54	4	82	189	110	66	138	52
26	1	55	7	83	139	111	117	139	43
27	1	56	3	84	101	112	40	142	73
28	1	57	3	85	105	113	45	144	9
29	1	58	7	86	101	114	70	146	0
30	0.4	59	7	87	84	115	66	149	0
31	0.3	60	4	88	75	116	59	151	0
32	1	61	8	89	72	117	25	153	0
33	1	62	11	90	77	118	37	156–7	0
34	1	63	8	91	90	119	35	163–4	0
35	1	64	28	92	101	120	36	170–1	0
36	2	65	26	93	112	121	73	177–8	0
37	1	66	107	94	93	122	87	191–2	0
38–39	1	67	63	95	41	123	111	212–3	0
								219	0

EPD = eggs per day. N = negative for eggs.

Rabbit 103. Figure 1 shows the egg counts (as moving 3-day averages of daily counts) which resulted from a series of 18 oral infections. The changes in the total number of eggs passed per day are seen to occur in weekly periods or cycles starting on or a day or two before the 14th day after each infection (which is the period required for the larvae of *T. calcaratus* to develop into adult worms and produce eggs). The first 8 doses of larvae produced rises in the egg count roughly proportional to the number of larvae given, but then, despite

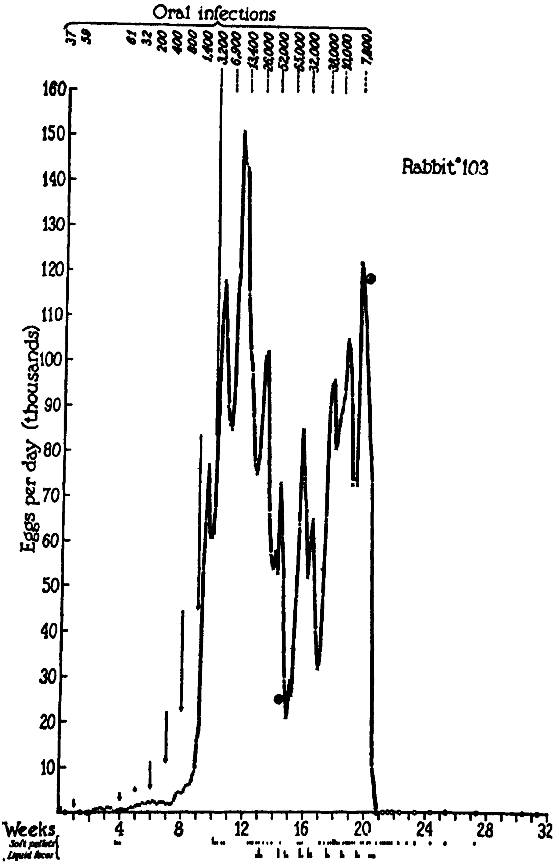


FIG. 1. Infestation record of Rabbit 103, showing initial susceptibility to infection, incomplete elimination of the infection followed by breaking through of the large doses of larvae, and finally complete elimination of the infection. Numbers in circles indicate adult worms recovered from feces. At bottom of chart are shown the association of passage of soft pellets with infection, and liquid feces with decrease in the egg count.

the giving of larger and larger doses, the count fell off precipitously in the 13th to 15th week. A second rise in the count occurred in the 16th week (evidently induced by the large 12th dose of 26,000 larvae) but was followed by a drop in the 17th week. The egg count mounted a third time in the 18th and 19th weeks, in evident response to the large 14th and 15th infections of 65,000 and 32,000 larvae, but dropped sharply to a negative in spite of two additional infections, and was consistently negative on 10 examinations during the following 11 weeks.

The reduced scale graph of this same infection (Figure 4) shows the contrast between the actual curve of egg production and the curve which would have been expected had the rabbit remained as susceptible to all the doses of larvae as it was to the first one. We interpret these egg counts as indicating that the rabbit was initially susceptible but that repeated infections developed a resistance which threw off the greater part of the worms. Some of the larger doses temporarily broke through this developing resistance. However, the rabbit not only failed to show a proportional increase in amount of infection when assaulted repeatedly with very large numbers of infective larvae, but finally completely rid itself of the infection.

The failure of the huge doses of larvae to produce comparable rises in Rabbit 103's egg count may be partly explained by the fact that a large proportion of the larvae passed directly through the animal during the two days following these infections. Thus 62 per cent of the 13th dose of larvae was recovered, as counted by dilution, 30 per cent of the 14th dose, 18 per cent of the 15th dose, and 21 per cent of the 16th dose. (Passage of infective larvae through the alimentary tract has been previously observed by Stoll (1929) when large doses of *Haemonchus contortus* larvae were fed to sheep.)

Rabbit 110. Figure 2 shows that the number of eggs passed by this rabbit increased with the number of larvae given for the first seven infections but then dropped off to a negative despite the larger and larger doses. Evidently a few worms from some of the last of these oral doses temporarily broke through the resistance which the animal had developed and produced a small egg count in the 18th and 19th weeks. A second series of 5 small oral doses, given to test the animal's resistance, produced only a delayed and barely detectable count.

The chart of this infection at a reduced scale (see Figure 4) shows the enormous disproportion between the amount of infection which the animal should have taken had it been uniformly susceptible and the amount which it actually acquired and then eliminated.

Rabbit 112. In this orally infected animal (Figure 3) only the first six doses of larvae added increments to the egg count which followed the larval curve, the count dropping to a negative in the 13th week after infection. A small count reappeared, however, and evidently the large 10th and 11th doses both broke through the rabbit's newly developed resistance to build up a high count in the 16th to 17th week. This secondary peak of infection was short lived but it left a residual infection (shown in Figure 3 by the dotted line at the 18th to 21st weeks) of about 10,000 eggs per day, upon which were superimposed 5 weekly doses *by skin* of between 500–2,000 larvae each. Apparently the count decreased during the time of these infections instead of increasing, showing that the animal was resistant to superimposed infection. At the termination of these test doses the infection established itself at a slightly higher level than before and remained there for some time. When the animal was examined a final time it had become negative, and no worms were found in the intestinal tract at autopsy six days later. The chart plotted at a reduced scale (see Figure 4) to show the complete theoretical curve of expected egg

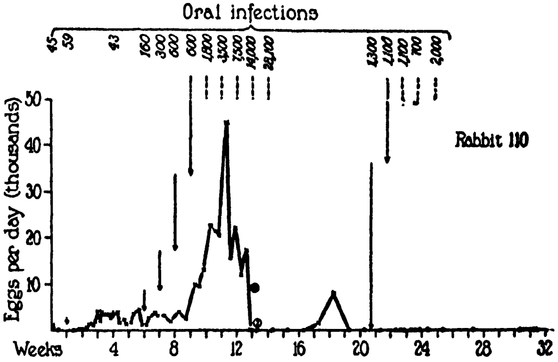


FIG. 2. Infestation record of Rabbit 110, showing initial susceptibility, occurrence of crisis and self-cure, and resistance to a second series of infections.

production illustrates the failure of the host to become parasitized by more than a very small fraction of the larvae which it received.

The gradual increase in the number of eggs passed in the dung of the 3 rabbits given an initial series of increasing infections by mouth, and the later abrupt drop in the egg count may be contrasted with the egg counts of Rabbit 116 (see Figure 5). This animal received a single dose of 2,500 *T. calcaratus* larvae by mouth at the time when a dose of approximately the same size and of the same larvae was given to

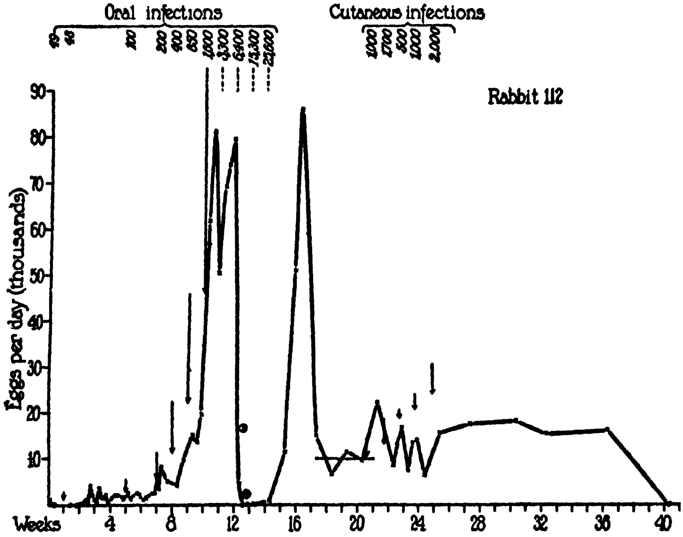


FIG. 3. Infestation record of Rabbit 112, showing initial susceptibility, self-cure, breaking through of heavy doses leaving a residual infection, relative immunity to a test series of skin infections, and ultimate elimination of the infection.

Rabbits 103, 110 and 112, as one of a series of doses, at 10 weeks after their first infection. In Rabbit 116, which had been found repeatedly negative for worm eggs from the time it was weaned until infected at four and one-half months of age, eggs appeared first on the 13th day and the count mounted very rapidly during the 3rd and 4th weeks to a peak of 108,000 eggs per day, and then declined gradually over a period of nearly a year, becoming permanently negative 49 weeks after infection. (When the decreasing egg counts of this animal were plotted on arith-logarithmic paper they approximated a straight line, from which the smooth curve $Y = 156{,}000\,(.86)^x$ of Figure 5 was fitted graphically, indicating that the infection was being lost at a constant rate.) This sharp rise in the egg count, and the following gradual decline are typical for single infections of this size. In the other 3 rabbits which received roughly the same doses, as only one of the smaller doses

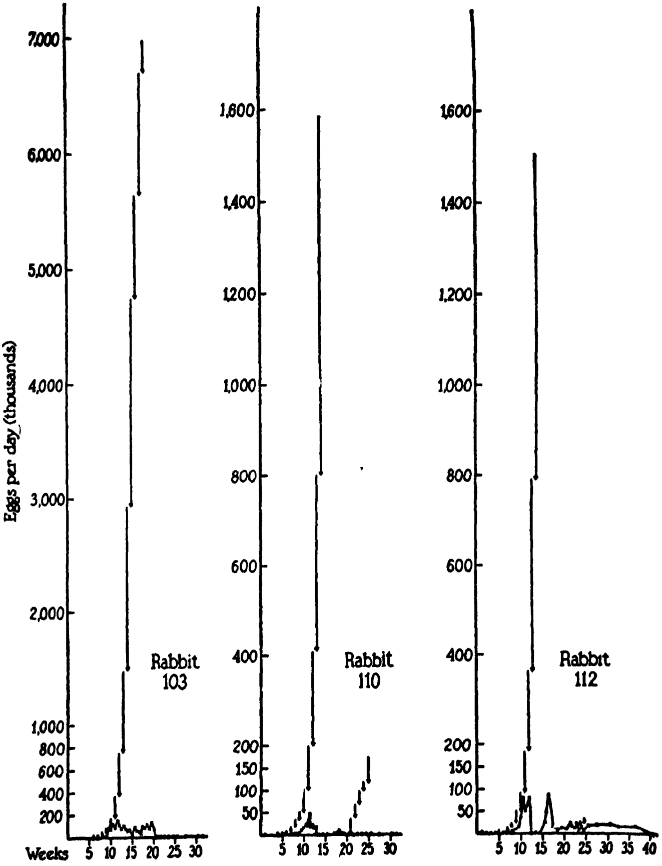


FIG. 4. Reduced scale graphs of the infections shown in Figures 1–3 contrasting the small size of the actual egg counts with the theoretical counts, indicated by the arrows, which would have resulted if the rabbits had remained as susceptible to all doses of larvae as they were to the first dose.

in their initial series of infections, there was no increase in count that could be attributed to this infection, and, on the contrary, the change in egg count which occurred in the 13th week, when eggs from this infection should have been expected to appear, was in every case a decrease, as may be seen in Figures 1, 2 and 3. The *whole series* of doses fed to Rabbits 103, 110 and 112 produced only in Rabbit 103 a count as high as that occasioned by the single dose given to Rabbit 116.

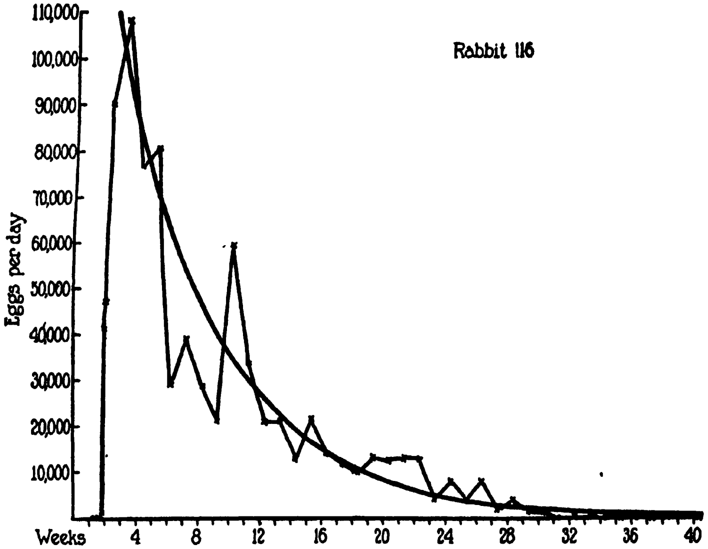


FIG. 5. Infestation produced by giving a previously uninfected rabbit a single oral infection of 2500 *T. calcaratus* larvae. Contrast the constant rate of loss of this infestation with the abrupt elimination of infestation in the 6 repeatedly infected rabbits.

Rabbit 104. This animal received 11 infections by skin (Figure 6), but due to a shortage of larvae it and the other two infected by skin received no doses of larvae for 5 weeks after the first two doses. There was very little increase in the small count arising from the first two infections until the sixth infection was given. This dose and the next one were followed by proportional increases in the egg count, which then dropped sharply to a negative in spite of three final infections of large numbers of larvae. The animal was autopsied the day after it became negative, and only 17 adult *T. calcaratus* were found although the rabbit had received a total of 38,000 larvae. If the animal had remained uniformly susceptible throughout the experiment the egg count would have reached the height shown by the arrows.

Rabbit 111. The number of worms colonizing the intestines of this animal, as indicated by the egg count (Figure 7), increased with the number of larvae given at first, then lagged behind, and finally dropped to a negative in the 16th week. The two final doses of the first series were evidently responsible for the slight temporary increase in the count during the 17th and 18th weeks. Five weekly doses of larvae given *orally* as a crucial test of this animal's resistance produced only a delayed and barely detectable egg count, whereas in a previously uninfected animal the count shown by the arrows would have been expected. This rabbit was the least susceptible to repeated infection

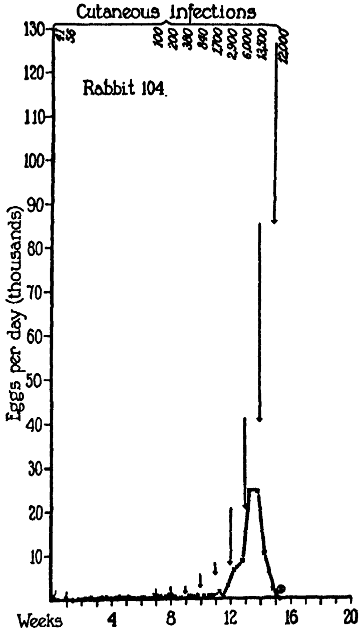


FIG. 6. Infestation record of Rabbit 104, showing occurrence of self-cure following repeated cutaneous infection.

of any of the 6 animals and demonstrated conclusively that a resistance acquired against skin infection also provides effective protection against mouth infection.

Rabbit 113. The first skin infection given this animal apparently did not induce any detectable infection, but the second to eighth doses inclusive produced proportional egg count increases (Figure 8). In contrast to this, the final three doses were followed by a rapid decrease in the count to a negative. Some of the larvae of these last three infections were probably responsible for the small and delayed count which appeared in the 20th, 21st and 22nd weeks. The resistance which had thus manifested itself was tested by a second series of 5 small weekly doses of larvae also given by skin. The egg count decreased while these were being given. The two transient increases which oc-

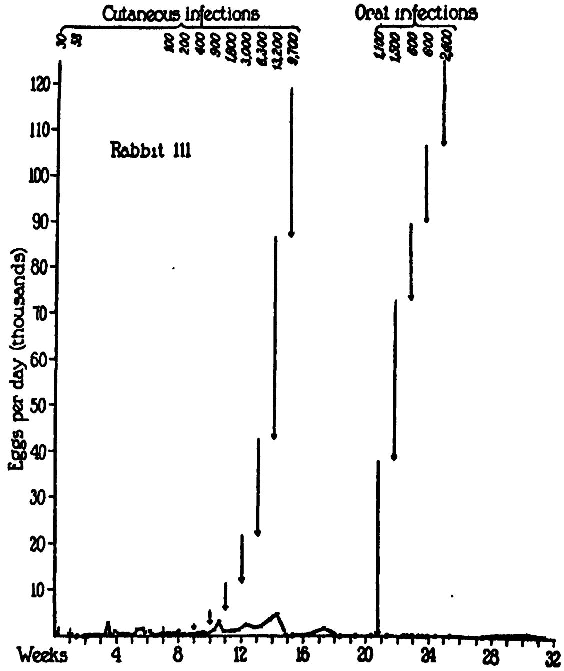


FIG. 7. Infestation record of Rabbit 111, showing early development of resistance to cutaneous infection and resulting refractoriness to oral infection.

curred following this second series of "test" doses may be interpreted as the breaking through of an infection followed by reestablishment of the animal's resistance.

Worms recovered from feces. Five of the 6 repeatedly infected rabbits passed adult worms in their dung at the time when the sharp drop occurred in their egg counts, proving that the decrease in the count was actually associated with loss of parasites. The worms were recovered by screening the crushed feces through screens of 20, 40 and 60 meshes to the inch. The total number of worms calculated to have been passed by each rabbit on each day that worms were recovered is shown by the numbers in circles in Figures 1 to 3 and Figures 6 to 8. These worms, recovered by the occasional screening of a comparatively small portion of the total feces passed by the rabbits, obviously represent only part of the total number of parasites which must

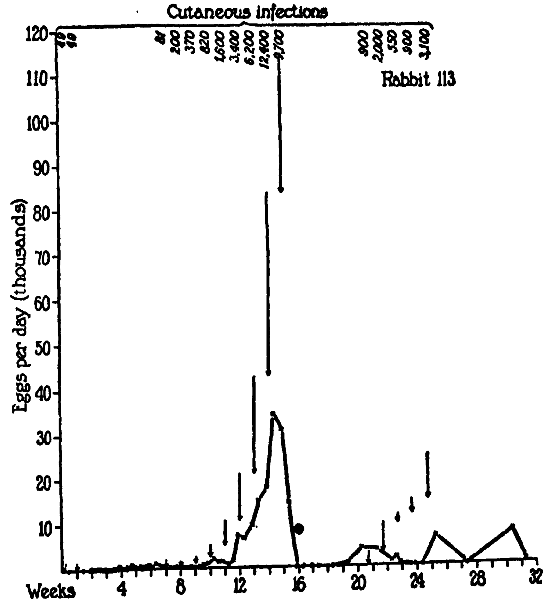


FIG. 8. Infestation record of Rabbit 113 illustrating the acquirement of resistance to superinfection, occurrence of self-cure, and relative resistance to a second series of infections.

have been eliminated to account for the great decreases in egg counts which occurred at the time the worms were found. That this passage of worms is not merely evidence of a normal turnover in the parasitic population of an animal exposed to repeated infection is shown by failure to find worms immediately before and after these sudden drops in the egg count, and by the more detailed data concerning Rabbit 103. At least 10 per cent of the dung passed each day by this rabbit from the 88th through the 101st day after its first infection was screened for worms but none were found. For this interval the egg count averaged 74,000 eggs per day. But when the egg count dropped on the 104th day to 26,500 eggs per day on a 102 gram specimen, of which 31 grams were liquid, 6 worms were recovered from 10 grams of the mixture, giving an estimated number of 60 worms in the total specimen.

Passage of soft pellets and liquid feces. Parasitic infection and the development of self-cure and resistance in the rabbits were associated with the passage of soft pellets and liquid feces. This is best shown by daily information secured on Rabbit 103. At the bottom of Figure 1 are indicated the days on which feces of either of these two consistencies were found in addition to the usual formed pellets. The short bars indicate that a small amount of the day's fecal specimen was of the designated form, the large ones that a relatively large proportion was passed. Before infection, and throughout the first 10 weeks after infection, the animal passed firm fecal pellets almost exclusively, but from the 10th week on masses of soft pellets occurred very frequently and diarrheic feces were observed every week from the 14th through the 21st. The worms which were recovered by screening Rabbit 103's feces were all from liquid specimens. There is not as detailed a record for the other five rabbits, but they, too, show a correlation of soft pellets and diarrheic feces with infection and loss of worms. This change in fecal consistency was not caused by diet, since it did not occur in the control rabbit, nor can it be laid to coccidial infection, as the animals had ceased to pass oocysts. Stoll (1929) observed a similar passage of soft pellets by sheep at the height of *Haemonchus contortus* infections.

Skin lesions. The only other clinical sign of the rabbits' reaction to repeated infection (their weight curves were not significantly different from that of the uninfected control) was the appearance of small nodules at the site of infection following some of the larger doses given by skin. Rabbit 104 and Rabbit 111 developed nodules after the 7th, 9th, 10th, and 11th skin infection, and Rabbit 113 showed them after the 9th and 10th doses of the first series, and after the 3rd and 4th test doses. They appeared within a few days after infection as papules of 2 to 5 mm. in diameter. They were firm, movable, yellow in the center, circumscribed with a narrow red zone, and blanched on pressure. They decreased in size for 2 or 3 weeks, becoming dry and white, and finally sloughed away leaving a pigmented spot in the skin.

DISCUSSION

Although the most striking characteristic of the infections produced in the 6 rabbits given increasing weekly doses of larvae may seem at first to be their dissimilarity, careful study of Figures 1 to 3 and Figures 6 to 8 will show that the general course of the infection was the same in each animal and that the differences were merely a matter of degree. All 6 were initially susceptible as shown by the establishment of an infection and its increase with the increase in the doses of larvae. All became resistant to superinfection after 6 to 8 doses of larvae, and this phase of their immunity development was uniformly followed by an abrupt drop in their egg counts associated with the passage of worms in their feces. In each animal this process of self-cure was followed by the temporary establishment of a new infestation resulting from the thousands of larvae which were being given at the culmination of the first series of doses. These secondary peaks of infection were in turn more or less completely reduced and the resistance reestablished as shown by the rabbits' relative resistance to a second series of doses. The increase of the egg count to a maximum occurred at the same time in all of the animals infected by the same method, although later in those infected by skin (first peak of infection at the 14–15th week) then in those infected by mouth (first peak of infection at the 11–12th week) because of the five week period when no larvae were given to the former group. The first sudden decrease in the egg count curves, indicating the occurrence of self-cure, also took place at roughly the same time in all rabbits of each group, occurring in the 12–15th week in the orally infected animals and in the 15–16th week in the cutaneously infected ones.

Obvious differences between the infections of the 6 repeatedly infected rabbits are shown in the general shape of the egg count graphs, and in the size of the egg counts. Since the number of larvae received by each rabbit was not the same the latter are better compared in terms of the egg-larva ratio, i.e. by a ratio obtained by dividing the egg count produced by a particular dose or group of doses by the number of larvae which had been given a sufficient time previously (at least two weeks) to have played a possible part. Table 3 gives such egg-larva ratios for each animal and also summarizes other comparative data. It will be seen from this table that infections given by skin took longer to appear than those given by mouth. The egg-larva ratio calculated for the total ascending portion of the egg count curves, or period of initial susceptibility, was 41 for oral infections and only 4.4 for cutaneous infections. The egg-larva ratio for Rabbit 116, given a single dose of 2,500 larvae, was 43. Thus during the early phase of the infection repeated doses of larvae were equally as effective in establishing worms in the three orally infected rabbits as a large dose of larvae given all at once to another animal.

The egg-larva ratios for individual animals during this same period show that Rabbit 110 was less susceptible to infection than the other two orally infected rabbits and Rabbit 111 less susceptible than the other two infected cutaneously. It is of interest that the later behavior of the rabbits is in accordance with their comparative susceptibility classification. Thus Rabbits 103 and 112 were more susceptible, on the basis of these ratios, than Rabbit 110, and in both the heaviest doses of the initial series of infections broke through their partially established resistance and succeeded in producing large infestations which were only thrown off after some time, whereas in Rabbit 110, the heavy doses of larvae succeeded only in reestablishing a small infection which was rapidly and completely eliminated. Likewise, among the skin infections, Rabbit 111, which had the lowest ratio and appeared to become resistant very early, showed a slightly and more transient breaking through of the largest doses of larvae than the more susceptible (by ratio) Rabbit 113, and also proved less susceptible to the test infections.

The egg-larva ratio may also be used to contrast the large total number of larvae which the rabbits received in their initial series of doses with the small amount of infection which was left at the time of the test doses. As Table 3 shows, 4 of the 6 rabbits had a zero ratio at this time, being negative for worm eggs, and the other two had a ratio of below 0.2 eggs per day per larva. The most striking case is that of Rabbit 103. This animal had been highly susceptible initially as shown by a ratio of 50 eggs per day per larva for the first 8 infections, totalling 3,000 larvae, but its ratio became zero in the 3rd week after its last infection of 7,800 larvae, although it had received a total of 257,000 larvae.

The purpose of the second series of doses which were given to 4 of the rabbits was to test the resistance to infection which the animals were believed to have acquired as a result of exposure to their initial series of infections and also in 2 rabbits to test for immunity against infection by the alternate method from that previously used. The degree to which the rabbits were susceptible to this second series of doses is shown in Table 3, in terms of egg-larva ratios based on the maximum increase which was observed in the egg counts. The five test doses given orally to Rabbit 110, which had become negative following a previous series of oral doses, gave a ratio of only 0.02. A similar series of oral doses given to Rabbit 111, which was negative as the result of an initial series of cutaneous doses, gave an egg-larva ratio of only 0.1. These two factors show that both these animals were relatively resistant to infection, since they represent the establishment of an insignificant egg count when compared with the average ratio of 41 for animals susceptible to oral infection. In Rabbit 113, which had eliminated most of its original infestation produced by cutaneous infection, the egg-larva ratio for the maximum increase in egg count produced by the test doses given cutaneously was 0.4. For Rabbit 112, carrying a residual infestation of uncertain level from an initial series of oral doses, the egg count decreased during the time that the test doses were being given by skin, but increased later to a new plateau of infection, which, if brought about solely by the test doses, would give a ratio of 1.3. As the ratio for animals susceptible to infection by skin was 4.4, both rabbits were relatively resistant by this test, as the increase brought about by the test doses was very slight in the case of Rabbit 113, and small, even if solely due to the test doses, in Rabbit 112. Of course any ratio above zero resulting from the test doses,

TABLE 3

Comparative Data Concerning Infestations of Rabbits Repeatedly Infected with Trichostrongylus calcaratus

	Initial Series of Infections									Test Infections			
Rabbit No.	Infected	1st Eggs (Days)	1st Peak (EPD)	No. doses in-volved	Total larvae given	Egg-larva ratio	Residual count (EPD)	Total larvae given	Egg-larva ratio	Infected	Total larvae given	Max. increase (EPD)	Egg-larva ratio
103	by mouth	15	151,000	8	3,000	50	0	257,000	0	....	.....	.....	....
110	" "	13	45,000	7	1,800	25	0	57,000	0	by mouth	6,200	100	0.02
112	" "	15	81,000	6	1,650	49	10,000	54,000	0.2	by skin	6,250	8,000	1.3
Average for mouth infections		14.3	94,000			41							
104	by skin	16	24,000	7	3,300	7.3	0	38,000	0	....	.....	.....	....
111	" "	16	5,000	8	6,500	0.8	0	36,000	0	by mouth	6,200	800	0.1
113	" "	26	34,000	8	6,600	5.1	4,000	35,000	0.1	by skin	7,450	3,000	0.4
Average for skin infections		19.3	21,000			4.4							

EPD = eggs per day.

although small in size when compared with the ratio for susceptible animals, is significant in that it demonstrates that the resistance of the rabbits was relative, not absolute. The resistance displayed by Rabbits 111 and 112 to infection by the alternative method from that to which they had previously become resistant is most marked in the case of Rabbit 111, but sufficiently definite in both animals to demonstrate that the protection is not limited to infection by the route used to produce the resistance.

The increasing age of the rabbits during the experiment is not considered to have played any significant part in their development of a resistance to infection since we have failed so far to demonstrate the presence of any marked age resistance in rabbits to infection with *T. calcaratus*.

The occurrence of self-cure and protection in rabbits exposed to repeated infection with *T. calcaratus*, as reported in the present paper has also been observed in a second series of rabbits given larger increasing doses of larvae, as well as in several groups of rabbits exposed to repeated infection out-of-doors.

The exact mechanism of the phenomenon has not been determined but the following points support the idea that this resistance is primarily a host reaction: the passage of soft pellets and diarrheic feces concomitantly with the loss of worms; the appearance of nodules in the skin after repeated cutaneous infection; the refractoriness to infection of rabbits which were originally susceptible to infection but have lost their worms; and the occurrence of self-cure in rabbits carrying infestations of greatly different magnitudes. The last two points would appear to furnish adequate proof that self-cure and resistance in rabbits is not related to mere parasitic overcrowding, since rabbits may be immune to infection although carrying no worms, and can discharge their worms although only few are present. The process appears then to be clearly analogous if not homologous to that observed by Stoll (1929) in sheep exposed to repeated infection with a stomach worm, *Haemonchus contortus*.

SUMMARY

Weekly increasing doses of the infective larvae of an intestinal nematode, *Trichostrongylus calcaratus*, were given to laboratory rabbits

by mouth and by skin, and the resulting infections followed by frequent egg counts.

All of the rabbits were susceptible to infection at first as shown by increases in the egg counts roughly proportional to the number of larvae given, but after 6 to 8 weekly doses of larvae they became refractory to superimposed infection and finally discharged the worms which they did harbor. This self-cure manifested itself by sharp drops in the egg counts associated with passage of soft pellets and liquid feces containing adult worms.

The rabbits as tested by a later second series of infections were found to possess a high degree of acquired resistance. Those previously infected by mouth were resistant to later infection by mouth and by skin, and those infected initially by skin were also resistant to infection by either method.

In contrast to the sudden loss of infection from rabbits given repeated doses of larvae, the infection produced in a rabbit given a single dose of larvae showed a constant rate of loss over a period of 49 weeks, as determined by egg counts.

ACKNOWLEDGMENT

The author is indebted to Dr. Norman R. Stoll for help in planning and starting the experiments and for assistance in the analysis and preparation of the results for publication.

REFERENCES CITED

Africa, C. M. 1931.—Studies on the host relations of *Nippostrongylus muris*, with special reference to age resistance and acquired immunity. Jour. Parasit., 18: 1–13.

Chandler, A. C. 1932.—Susceptibility and resistance to helminthic infections. Jour. Parasit., 18: 135–152.

Cort, W. W., Ackert, J. E., Augustine, D. L., and Payne, F. K. 1922.—Investigations on the control of hookworm disease. II. The description of an apparatus for isolating infective hookworm larvae from soil. Amer. Jour. Hyg., 2: 1–16.

Fülleborn, F. 1926.—Ueber das Verhalten der Hakenwurmlarven bei der Infektion per os. Arch. Schiffs- Trop.-Hyg., 30: 638–653.

Graham, G. L., Ackert, J. E., and Jones, R. W. 1932.—Studies on an acquired resistance of chickens to the nematode, *Ascaridia lineata* (Schneider). Amer. Jour. Hyg., 15: 726–740.

Herrick, C. A. 1928.—A quantitative study of infections with *Ancylostoma caninum* in dogs. Amer. Jour. Hyg., 8: 125–157.

McCoy, O. R. 1931a.—Immunity reactions of the dog against hookworm (*Ancylostoma caninum*) under conditions of repeated infection. Amer. Jour. Hyg., 14: 268–303.

1931b.—Immunity of rats to reinfection with *Trichinella spiralis*. Amer. Jour. Hyg., 14: 484–494.

Payne, F. K. 1923.—Investigations on the control of hookworm disease, XXXI. The relation of the physiological age of hookworm larvae to their ability to infect the human host. Amer. Jour. Hyg., 3: 584–597.

Ransom, B. H. 1911.—Two new species of parasitic nematodes. Proceed. U. S. Nat. Museum, 41: 363–369.

Sandground, J. H. 1928.—Some studies on susceptibility, resistance, and acquired immunity to infection with *Strongyloides stercoralis* (Nematoda) in dogs and cats. Amer. Jour. Hyg., 8: 507–538.

Sarles, M. P. 1929.—The length of life and rate of loss of the dog hookworm, *Ancylostoma caninum*. Amer. Jour. Hyg., 10: 667–682.

1930.—The occurrence of self-cure and protection in rabbits receiving repeated infections of *Trichostrongylus calcaratus* Ransom. Jour. Parasit., 17: 114.

Schwartz, B., Alicata, J. E., and Lucker, J. T. 1931.—Resistance of rats to superinfection with a nematode, *Nippostrongylus muris*, and an apparently similar resistance of horses to superinfection with nematodes. Jour. Wash. Acad. Sc., 21: 259–261.

Scott, J. A. 1928.—An experimental study of the development of *Ancylostoma caninum* in normal and abnormal hosts. Amer. Jour. Hyg., 8: 158–204.

Stoll, N. R. 1929.—Studies with the strongyloid nematode, *Haemonchus contortus*. I. Acquired resistance of hosts under natural reinfection conditions out-of-doors. Amer. Jour. Hyg., 10: 384–418.

1930.—On methods of counting nematode ova in sheep dung. Parasit., 22: 116–136.

1932.—Note on re-infection under "natural" conditions with a gut nematode of the rabbit. Jour. Parasit., 19: 54–60.

INDEX TO AUTHORS

ALVING, Alf. See Page and Alving, 311

—. See Van Slyke, Alving, and Rose, 329

Anson, M. L., and Mirsky, A. E. The estimation of pepsin with hemoglobin, 253

Anthony, Albert J., Cohn, Alfred E., and Steele, J. Murray. Studies of the influence of pulmonary motion and distention on movements of the thorax. I. Chemical and nervous factors involved in establishing apnea. II. The relation between pulmonary and thoracic movements, 445

—, —, and —. Studies on Cheyne-Stokes respiration, 465

BLINKS, L. R. Protoplasmic potentials in *Halicystis*. II. The effects of potassium on two species with different saps, 259

CAIN, C. B. See Seifried and Cain, 489, 495

Casey, Albert E., and Rosahn, Paul D. Supravital differential counting adapted to clinical use, 57

Cohn, Alfred E., and Steele, J. Murray. Studies on the effect of the action of digitalis on the output of blood from the heart. I. The effect on the output of the dog's heart in heart-lung preparations, 345

—. See Anthony, Cohn, and Steele, 445, 465

Cohn, Alfred E. See Stewart and Cohn, 375, 397

Cortese, Frank. See Levene and Cortese, 157

DICK, Macdonald. See Smith and Dick, 1

Dubos, René. See Goodner and Dubos, 293

Duran-Reynals, F. See Sturm and Duran-Reynals, 77

ELDERFIELD, Robert C. See Jacobs and Elderfield, 171

FAVILLI, Giovanni. The influence of some organ extracts on the permeability to water of sea urchin eggs (*Arbacia punctulata*), 47

Flexner, Simon. Immunity to human and passage poliomyelitis virus, 85

GOODNER, Kenneth, and Dubos, René. Studies on the quantitative action of a specific enzyme in Type III pneumococcus dermal infection in rabbits, 293

HARRIS, Stanton A. See Levene and Harris, 149

JACOBS, Walter A. The ergot alkaloids. I. The oxidation of ergotinine, 183

— and Elderfield, Robert C. Strophanthin. XXVII. Ring III of strophanthidin and related aglucones, 171

LANDSTEINER, K., and van der Scheer, J. Serological reactions with simple chemical compounds (precipitin reactions), 21

Levene, P. A., and Cortese, Frank. A note on the use of 1-bromotetramethylglucose for the synthesis of methylated glycosides, 157

— and Harris, Stanton A. The ribosephosphoric acid from xanthylic acid. II, 149

— and Marker, R. E. Maximum rotations and correlation of disubstituted acetic acids containing a methyl group, 141

— and Raymond, Albert L. On the structure of the so called 5-methylglucose of Ohle and von Vargha, 115

— and —. The substitution of glucose in position (4). I, 127

— and Steiger, Robert E. Studies on racemization. XI. Action of alkali on polypeptides, 103

—, —, and Rothen, Alexandre. The relation of chemical structure to the rate of hydrolysis of peptides. VII. Hydrolysis of dipeptides by alkali, 93

— and Walti, A. Phytochemical reduction of 1-hydroxy-2-oxo-heptane (heptanol-1-one-2), 167

—. See Lipmann and Levene, 161

Lipmann, Fritz A., and Levene, P. A. Serinephosphoric acid obtained on hydrolysis of vitellinic acid, 161

MARKER, R. E. See Levene and Marker, 141

Michaelis, L. Ein Reduktions-Indikator im Potentialbereich der Wasserstoffüberspannung, 277

Miller, Franklin R. The induced development of non-acid-fast forms of *Bacillus tuberculosis* and other mycobacteria, 33

Mirsky, A. E. See Anson and Mirsky, 253

Murphy, James B., and Sturm, Ernest. Properties of the causative agent of a chicken tumor. VI. Action of the associated inhibitor on mouse tumors, 61

— and —. VII. Separation of the associated inhibitor from tumor extracts, 71

NORTHROP, John H. Crystalline pepsin, 197

—. Crystalline pepsin. IV. Hydrolysis and inactivation by acid, 227

—. Pepsin activity units and methods for determining peptic activity, 235

OSTERHOUT, W. J. V. The kinetics of penetration. IV. Diffusion against a growing potential gradient in models, 269

PAGE, Irvine H., and Alving, Alf. The effect of nasopharyngeal operations on renal function, 311

— and Van Slyke, Donald D. A simple test for plasma protein contents below the edema-producing level, 343

Parker, Raymond C. The functional characteristics of nine races of fibroblasts, 189

—. The stability of functionally distinct races of fibroblasts, 193

RAYMOND, Albert L. See Levene and Raymond, 115, 127

Rosahn, Paul D. See Casey and Rosahn, 57

Rose, W. B. See Van Slyke, Alving, and Rose, 329

Rothen, Alexandre. See Levene, Steiger, and Rothen, 93

SARLES, MERRITT P. Development of an acquired resistance in rabbits by repeated infection with an intestinal nematode, *Trichostrongylus calcaratus* Ransom, 1911, 545

SCHUBERT, MAXWELL. Complex types involved in the catalytic oxidation of thiol acids, 281

SEIFRIED, O., and CAIN, C. B. Histological studies on hog cholera. II. Lesions of the vascular system, 489

— and —. III. Lesions in the various organs, 495

SHOPE, RICHARD E. Studies on immunity to swine influenza, 507

SMITH, FREDERICK, and DICK, MACDONALD. The influence of the plasma colloids on the gradient of capillary permeability, 1

SPRUNT, DOUGLAS H. Infectious myxomatosis (Sanarelli) in pregnant rabbits, 303

STEELE, J. MURRAY. See ANTHONY, COHN, and STEELE, 445, 465

—. See COHN and STEELE, 345

STEIGER, ROBERT E. See LEVENE and STEIGER, 103

—. See LEVENE, STEIGER, and ROTHEN, 93

STEWART, HAROLD J., and COHN, ALFRED E. Studies on the effect of the action of digitalis on the output of blood from the heart. II. The effect on the output of the hearts of dogs subject to artificial auricular fibrillation, 375

STEWART, HAROLD J., and COHN, ALFRED E. III. Part 1. The effect on the output in normal human hearts. Part 2. The effect on the output of hearts in heart failure with congestion, in human beings, 397

STOLL, NORMAN R. Note on reinfection under "natural" conditions with a gut nematode of the rabbit, 519

—. Studies with the strongyloid nematode, *Haemonchus contortus*. II. Potential infestation curves under conditions of natural reinfection, 527

STURM, ERNEST, and DURAN-REYNALS, F. Properties of the causative agent of a chicken tumor. VIII. The effect of testicle extract on the rate of growth of Chicken Tumor I, 77

—. See MURPHY and STURM, 61, 71

VAN DER SCHEER, J. See LANDSTEINER and VAN DER SCHEER, 21

VAN SLYKE, DONALD D., ALVING, ALF, and ROSE, W. B. Studies of urea excretion. VII. The effects of posture and exercise on urea excretion, 329

—. See PAGE and VAN SLYKE, 343

WALTI, A. See LEVENE and WALTI, 167

INDEX TO SUBJECTS

ACETIC acids, disubstituted, with methyl group, rotations, correlation, 141
Acid fastness, *Bacillus tuberculosis*, induced development of non-acid-fast forms, 33
— —, mycobacteria, induced development of non-acid-fast forms, 33
Aglucones, strophanthidin-related, Ring III, 171
Alkali, hydrolysis of dipeptides, 93
—, racemization effect on polypeptides, 103
Alkaloids, ergot, 183
Apnea, chemical and nervous factors involved in establishing, 445
Arbacia punctulata eggs, permeability to water, influence of organ extracts, 47
Auricular fibrillation, artificial, effect of digitalis on output of hearts of dogs subject to, 375

BACILLUS *tuberculosis*, non-acid-fast forms, induced development, 33
Bacteria, myco-, induced development of non-acid-fast forms, 33
Blood counts, differential, supravital, adapted to clinical use, 57
—, output from heart, effect of action of digitalis, 345, 375, 397
— vessel, influence of plasma colloids on gradient of capillary permeability, 1
— —, lesions in hog cholera, 489
1-Bromotetramethylglucose, use in synthesis of methylated glycosides, 157

CAPILLARY permeability, gradient, influence of plasma colloids, 1
Catalytic oxidation of thiol acids, complex types involved, 281
Chemical compounds, simple, precipitin reactions, 21
— —, —, serological reactions, 21
— constitution, glucose, position (4), 127
— —, 5-methylglucose, Ohle and von Vargha, 115
— —, peptide hydrolysis rate, relation, 93
— —, strophanthidin, Ring III, 171
— —, strophanthidin-related aglucones, Ring III, 171
— and nervous factors involved in establishing apnea, 445
Cheyne-Stokes respiration, 465
Cholera, hog, histology, 489, 495
—, —, —, lesions in various organs, 495
—, —, —, — of vascular system, 489
Colloids, plasma, influence on gradient of capillary permeability, 1

DIGITALIS action, effect on output of blood from heart, 345, 375, 397
Dipeptides, alkali hydrolysis, 93

EDEMA, simple test for plasma protein contents below level producing, 343
Eggs, *Arbacia punctulata*, permeability to water, influence of organ extracts, 47

Eggs, sea urchin, permeability to water, influence of organ extracts, 47
Enzyme, specific, quantitative action in Type III pneumococcus dermal infection, 293
Ergot alkaloids, 183
Ergotinine, oxidation, 183
Excretion, urea, 329
—, —, effects of exercise, 329
—, —, — — posture, 329
Exercise, effects on urea excretion, 329
Extract, testicle, effect on rate of growth of Chicken Tumor I, 77
Extracts, organ, influence on permeability to water of sea urchin eggs, *Arbacia punctulata*, 47
—, tumor, separation of associated inhibitor, 71

FIBRILLATION, auricular, artificial, effect of digitalis on output of hearts of dogs subject to, 375
Fibroblasts, functionally distinct races, stability, 193
—, nine races, functional characteristics, 189
Fowl, tumor, action of associated inhibitor on mouse tumors, 61
—, —, extracts, separation of associated inhibitor, 71
—, —, properties of causative agent, 61, 71, 77
—, —, rate of growth, effect of testicle extract, 77

GLUCOSE, 1-bromotetramethyl-, use in synthesis of methylated glycosides, 157
—, 5-methyl-, Ohle and von Vargha, chemical constitution, 115
—, substitution, position (4), 127
Glycosides, methylated, synthesis, 1-bromotetramethylglucose for, 157

HAEMONCHUS *contortus*, strongyloid nematode, potential infestation curves under conditions of natural reinfection, 527
Halicystis, protoplasmic potentials, 259
Heart, dog, effect of digitalis on output in heart-lung preparations, 345
— failure with congestion in human beings, effect of digitalis on output of hearts, 397
—, human, in heart failure with congestion, effect of digitalis on output, 397
—, —, normal, effect of digitalis on output, 397
— of dogs subject to artificial auricular fibrillation, effect of digitalis on output, 375
—, output of blood, effect of action of digitalis, 345, 375, 397
Heart-lung preparations, effect of digitalis on output of dog's heart in, 345
Hemoglobin, estimation of pepsin with, 253
Heptane, 1-hydroxy-2-oxo-, reduction, phytochemical, 167
Heptanol-1-one-2, reduction, phytochemical, 167
Histology, hog cholera, 489, 495
—, — —, lesions in various organs, 495
—, — —, — of vascular system, 489
Hog cholera. *See* Cholera.
Hydrogen overvoltage, potential range, oxidation-reduction indicator, 277
1-Hydroxy-2-oxo-heptane, reduction, phytochemical, 167

IMMUNITY to human and passage poliomyelitis virus, 85
— — swine influenza, 507
Influenza, swine, immunity, 507

Intestinal nematode of rabbit, reinfection under natural conditions, 519
— —, *Trichostrongylus calcaratus* Ransom, 1911, development of acquired resistance in rabbits by repeated infection with, 545

KIDNEY function, effect of nasopharyngeal operations, 311
Kinetics of penetration, 269

LUNG-HEART preparations, effect of digitalis on output of dog's heart in, 345

5-METHYLGLUCOSE, Ohle and von Vargha, chemical constitution, 115
Mycobacteria, non-acid-fast forms, induced development, 33
Myxomatosis, Sanarelli, infectious, in pregnant rabbits, 303

NASOPHARYNGEAL operations, effect on renal function, 311
Nematode, intestinal, of rabbit, reinfection under natural conditions, 519
—, —, *Trichostrongylus calcaratus* Ransom, 1911, development of acquired resistance in rabbits by repeated infection with, 545
—, strongyloid, *Haemonchus contortus*, potential infestation curves under conditions of natural reinfection, 527
Nervous and chemical factors involved in establishing apnea, 445

OHLE-VON VARGHA, 5-methylglucose, chemical constitution, 115
Optical rotation, acetic acids, disubstituted, with methyl group, 141
Organ extracts, influence on permeability to water of sea urchin eggs, *Arbacia punctulata*, 47
Oxidation, catalytic, of thiol acids, complex types involved, 281
—, ergotinine, 183
Oxidation-reduction indicator in potential range of hydrogen overvoltage, 277

PENETRATION, kinetics, 269
Pepsin activity units and methods for determining peptic activity, 235
—, crystalline, 197, 227
—, —, hydrolysis by acid, 227
—, —, inactivation by acid, 227
—, estimation with hemoglobin, 253
Peptic activity, methods for determining, and pepsin activity units, 235
Peptides, chemical constitution, hydrolysis rate, relation, 93
—, di-, alkali hydrolysis, 93
Phosphoric acid, ribose-, from xanthylic acid, formation, 149
Phytochemical reduction. *See* Reduction.
Plasma colloids, influence on gradient of capillary permeability, 1
— protein contents below edema-producing level, simple test, 343
Pneumococcus Type III, dermal infection, quantitative action of specific enzyme, 293
Poliomyelitis virus, human and passage, immunity to, 85
Polypeptides, racemization, alkali action, 103
Posture, effects on urea excretion, 329
Potassium, effect on two species of *Halicystis* with different saps, 259

Potential gradient, growing, in models, diffusion against, 269
Potentials, protoplasmic, in *Halicystis*, 259
Precipitin reactions, serological reactions with simple chemical compounds, 21
Pregnancy, infectious myxomatosis, Sanarelli, in rabbits, 303
Protein, plasma contents, below edema-producing level, simple test, 343
Protoplasm, potentials, in *Halicystis*, 259
Pulmonary motion and distention, influence on movements of thorax, 445
— and thoracic movements, relation, 445

RABBIT, acquired resistance developed by repeated infection with intestinal nematode, *Trichostrongylus calcaratus* Ransom, 1911, 545
—, reinfection under natural conditions with gut nematode, 519
Reactions, precipitin, serological reactions with simple chemical compounds, 21
Reduction, phytochemical, heptanol-1-one-2, 167
—, —, 1-hydroxy-2-oxo-heptane, 167
Reduction-oxidation indicator in potential range of hydrogen overvoltage, 277
Renal. *See* Kidney.
Resistance, acquired, development in rabbits by repeated infection with intestinal nematode, *Trichostrongylus calcaratus* Ransom, 1911, 545
Respiration, Cheyne-Stokes, 465
Ribosephosphoric acid from xanthylic acid, formation, 149

SAPS, different, of two species of *Halicystis*, effects of potassium, 259
Sea urchin eggs, permeability to water, influence of organ extracts, 47
Serinephosphoric acid, formation from vitellinic acid hydrolysis, 161
Serological reactions with simple chemical compounds, precipitin reactions, 21
Skin, pneumococcus infection, Type III, quantitative action of specific enzyme, 293
Specific enzyme, quantitative action in Type III pneumococcus dermal infection, 293
Strongyloid nematode, *Haemonchus contortus*, potential infestation curves under conditions of natural reinfection, 527
Strophanthidin, Ring III, 171
Strophanthidin-related substances, Ring III, 171
Strophanthin, 171
Swine influenza, immunity, 507

TESTICLE extract, effect on rate of growth of Chicken Tumor I, 77
Thiol acids, complex types involved in catalytic oxidation, 281
Thoracic and pulmonary movements, relation, 445
Thorax, movements, influence of pulmonary motion and distention, 445
Trichostrongylus calcaratus Ransom, 1911, intestinal nematode, development of acquired resistance in rabbits by repeated infection with, 545
Tuberculosis, bacillus, non-acid-fast forms, induced development, 33

Tumor, chicken, action of associated inhibitor on mouse tumors, 61
—, —, extracts, separation of associated inhibitor, 71
—, —, properties of causative agent, 61, 71, 77
—, —, rate of growth, effect of testicle extract, 77
—, mouse, action of associated inhibitor of chicken tumor on, 61

UREA excretion, 329
— —, effects of exercise, 329
— —, — — posture, 329

VIRUS, poliomyelitis, human and passage, immunity to, 85
Vitellinic acid, serinephosphoric acid formation from hydrolysis of, 161

WATER, influence of organ extracts on permeability of sea urchin eggs, *Arbacia punctulata*, 47

XANTHYLIC acid, ribosephosphoric acid from, 149